

11-30-00

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIFORNIA 94304

TELEPHONE 650-849-6600  
FACSIMILE 650-849-6666

WRITER'S DIRECT DIAL 650-849-6656  
JOHN.BURNS@FINNEGAN.COM

November 28, 2000

ATTORNEY DOCKET NO. 7414.0022  
CUSTOMER NUMBER: 22,852

**Box PATENT APPLICATION**  
**Assistant Commissioner for Patents**  
**Washington, DC 20231**

CERTIFICATE OF EXPRESS MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service's "Express Mail Post Office to Addressee" service under 37 CFR § 1.10, in an envelope addressed to: BOX PATENT APPLICATION, Assistant Commissioner For Patents, Washington, D.C. 20231, on November 28, 2000.

Express Mail Label No. **EL 589696257US**

Signed:

*Linda Phillips*  
Linda Phillips

New U.S. Patent Application

Title: COMPOSITIONS, METHODS, AND KITS FOR ISOLATING  
NUCLEIC ACIDS USING SURFACTANTS AND PROTEASES

Inventor: Lawrence Greenfield

Sir:

We enclose the following papers for filing in the United States Patent and Trademark Office in connection with the above patent application:

1. Application - 58 pages, including title page, 12 pages of claims [4 independent claims and 87 claims total], and abstract page;
2. Drawings - 14 sheets of drawings (Figures 1-12); and
3. Declaration and Power of Attorney (1 page).

Assistant Commissioner for Patents

November 28, 2000

Page 2

4. The filing fee is calculated as follows:

Basic Application Filing Fee					\$710	\$ 710.00
	Number of Claims		Basic	Extra Claims		
Total Claims	87	-	20	67	x \$18	\$1206.00
Independent Claims	4	-	3	1	x \$80	\$80.00
<input type="checkbox"/> Presentation of Multiple Dep. Claim(s)					+\$270	
Subtotal						\$ 1996.00
Reduction by 1/2 if small entity						-
TOTAL APPLICATION FILING FEE						\$ 1996.00

5. Please charge the filing fee of \$1996.00 to Deposit Account No. 06-0916. The fee includes:

\$710.00 filing fee; and  
\$1286.00 additional claims fee.

Please address all correspondence with respect to this application to:

Finnegan, Henderson, Farabow,  
Garrett & Dunner, L.L.P.  
1300 I Street, N.W.  
Washington, D.C. 20005-3315

Please accord this application an application number and filing date.

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

Assistant Commissioner for Patents

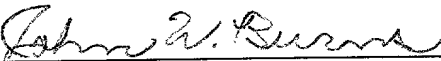
November 28, 2000

Page 3

The Commissioner is hereby authorized to charge any additional fees due and any other fees under 37 C.F.R. § 1.16 or § 1.17 during the pendency of this application to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,  
GARRETT & DUNNER, L.L.P.

By:   
John W. Burns  
Reg. No. 43,520

JWB/lp  
Enclosures

United States Patent Application

of

Lawrence Greenfield

for

Compositions, Methods, and Kits for Isolating Nucleic Acids Using  
Surfactants and Proteases

**EXPRESS MAIL CERTIFICATE**

"Express Mail" mail labeling  
number:

EL 589696257 US

Date of  
Deposit:

November 28, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to Box Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

Linda Phillips  
Printed Name

*Linda Phillips*  
Signature

**LAW OFFICES**

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Compositions, Methods, and Kits for Isolating Nucleic  
Acids Using Surfactants and Proteases

Field of the Invention

5           The invention relates to compositions and methods for releasing and for isolating nucleic acids from biological samples, preferably from whole tissue. The invention also provides kits for isolating and/or releasing nucleic acids from biological samples.

Background of the Invention

10           Current methods for isolating nucleic acids from cultured cells generally include lysing the cells and inactivating nucleases using chaotropic salts, such as guanidine hydrochloride or guanidinium thiocyanate, and a nonionic surfactant. The released nucleic acids are then selectively precipitated from solution. The combination of chaotropic salts and nonionic surfactants, however, typically are ineffective at penetrating whole tissues to release nucleic acid.

15           When cells are lysed to release nucleic acids, endogenous nucleases, enzymes that degrade nucleic acids (DNA and RNA), are also released. Thus, when minimally degraded (i.e., high integrity) nucleic acid is desired, one tries to minimize nuclease activity as much as possible. Deoxyribonucleases (DNAses), which are nucleases that degrade DNA, can often be inactivated by adding divalent cation chelators to the  
20           reaction compositions. Free magnesium ions, which are typically needed for DNase activity, are bound or complexed by the addition of chelators. Thus, chelators typically diminish or inactivate endogenous DNAses. Many endogenous ribonucleases (RNAses), nucleases that degrade RNA, however, do not typically need divalent

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

cations, such as magnesium, for their activity. Such RNAses are unaffected by the addition of chelators. To inactivate RNAses during RNA isolation procedures, therefore, one may add to the reaction composition inhibitors of RNase activity.

5 Various chemical disruption methods, using surfactants, chaotropes, proteases, bile salts, organic solvents, and harsh acidic or basic conditions have been employed to lyse cells and release nucleic acid. Proteinase K, a broad specificity protease, has also been employed. To increase the activity of Proteinase K, denaturing agents, such as anionic detergents and chaotropes have been used in combination with Proteinase K. The nucleic acid obtained using such methods may be degraded due to long incubation  
10 times or exposure to harsh conditions.

Physical disruption methods using tissue homogenizers, mortar and pestle, dounce homogenization, frozen pulverization, and bead beaters, have been employed with biological samples. The nucleic acid obtained using these methods, however, is typically degraded due to shearing, long incubation times and high temperatures that  
15 may be generated using these methods. Further, such methods present health and safety concerns as a portion of the sample may be aerosolized during the physical disruption procedure. This becomes a critical issue when the sample potentially contains infectious agents such as HIV, hepatitis, or other pathogenic microorganisms.

20 Thus, there is a need for methods and compositions for isolating high integrity, i.e., high molecular weight, nucleic acid molecules from biological samples in a safe, rapid, and efficient manner, especially from whole tissues.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

## Summary of the Invention

The present invention provides novel compositions, methods, and kits for releasing or for isolating nucleic acids from biological samples using at least one surfactant and at least one protease.

5 In one aspect, compositions are provided for releasing nucleic acids from a biological sample. In certain embodiments, these compositions include at least one cationic surfactant, at least one protease, and a buffer.

10 In another aspect, methods for releasing and methods for isolating nucleic acids from a biological sample are provided. In certain embodiments, these methods include combining the sample with at least one cationic surfactant, at least one protease, and a buffer to form a reaction composition. Optionally, the reaction composition further comprises a second surfactant and a salt. The composition is incubated under appropriate conditions to allow the sample to be digested, releasing the nucleic acid. In certain embodiments, the released nucleic acid is then isolated.

15 Kits for isolating and/or releasing nucleic acid from biological samples are also provided. In certain embodiments, the invention provides kits comprising at least one cationic surfactant and at least one protease. Kits further comprising a second surfactant, a salt, organic extraction agent(s), organic precipitating agent(s), solubilizing agents(s), or combinations of these components are also provided.

### LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Brief Description of the Figures

Figure 1: represents the fluorescent intensity (measured in fluorescence units) of dye-labeled casein substrate incubated with Proteinase K at concentrations ranging from 40 fg/mL to 40 ug/mL.

- 5 Figure 2: illustrates the effect of various surfactants on Proteinase K digestion of a dye-labeled casein substrate. Figure 2A depicts a reaction composition comprising 20 g/ml Proteinase K; Fig. 2B depicts a reaction composition comprising 2.5 g/ml Proteinase K; Fig. 2C depicts a reaction composition comprising 1.25 g/ml Proteinase K. The
- 10 tetraalkylammonium halide symbol (open diamond) represents the average data for the cationic surfactants dodecyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, hexadecyltrimethylammonium bromide, cetyltrimethylammonium bromide, and mixed alkyltrimethylammonium bromide. The symbol for benzylalkylammonium halide (open circle) represents the average data for the cationic surfactants benzalkonium
- 15 chloride, benzyldimethyldodecylammonium bromide, benzyldimethylhexadecylammonium bromide and benzyldimethyltetradecyl ammonium chloride. The symbol for Mackernium (X) represents the average data for the cationic surfactants Mackernium SDC-85, Mackernium KP, Mackernium WLE and Mackernium NLE. The polyquaternium symbol (closed circle) represents the average data for the cationic polymers Mackernium 006 and Mackernium 007. The results obtained using
- 20 water (closed diamond) and using the anionic surfactant SDS (closed square) are also shown. The error bars represent the standard deviation for the each data set.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600



Figure 3: illustrates the quantity of nucleic acid, measured in g of nucleic acid/mg of sample, released using reaction compositions comprising Proteinase K and various surfactants.

5 Figure 4: depicts a stained agarose gel that provides a comparison between the amount of nucleic acid that is released (supe) with the nucleic acid remaining in the sample (pellet) following incubation with the reaction compositions comprising the indicated surfactants.

10 Figure 5: illustrates the quantity of nucleic acid that is released and isolated from liver slices incubated for 20 minutes at 45° C in reaction compositions comprising 2 mg/mL Proteinase K and the indicated surfactants. When used in these figures, NMP is 1-methyl-2-pyrrolidinone.

Figure 6: depicts an ethidium bromide stained agarose gel that demonstrates the quality (integrity) of the released and isolated nucleic acid of Figure 5.

15 Figure 7: illustrates the quantity of nucleic acid that is released and isolated from liver slices incubated for 30 minutes at 45° C in reaction compositions comprising 1 mg/mL Proteinase K and the indicated surfactants and additives. When used in these figures, "nothing" means no additional reagent was added.

Figure 8: depicts an ethidium bromide stained agarose gel that demonstrates the quality (integrity) of the released and isolate nucleic acid of Figure 7.

20 Figure 9: illustrates the effect of exposing liver slices to pretreatment solutions prior to incubation for 30 minutes at 45° C in reaction compositions comprising 1 mg/mL

Proteinase K and the indicated surfactants and additives. When used in these figures, HTSP 2X means Nucleic Acid Purification Lysis Solution; AcrOr means acridine orange.

Figure 10: depicts a stained agarose gel that demonstrates the quality of nucleic acid released and isolated from samples that were incubated in pretreatment solutions prior to surfactant:Proteinase K treatment.

Figure 11: depicts a stained agarose gel that demonstrates the quality of nucleic acid released and isolated using reaction compositions comprising varying amounts of aurintricarboxylic acid.

Figure 12: depicts a stained agarose gel that provides a comparison of the amount and integrity of nucleic acid released and isolated with various cationic compounds.

#### Detailed Description of the Invention

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents cited in this application, including, but not limited to patents, patent applications, articles, books, and treatises, are expressly incorporated by reference in their entirety for any purpose.

#### Definitions

The term "alkyl group" refers to a hydrocarbon moiety based on a linear, branched or cyclic alkane. Linear alkanes are organic compounds with the general chemical formula  $C_nH_{2n+2}$ , where C represents a carbon atom, H represents a hydrogen atom, and n represents a whole number. Exemplary linear alkanes include, but are not limited to, methane ( $CH_4$ ), propane ( $C_3H_8$ ), butane ( $C_4H_{10}$ ), octane and the like.

Exemplary branched alkanes include, but are not limited to, isobutane ( $C_4H_{10}$ ),

20

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

isopentane (C<sub>5</sub>H<sub>12</sub>), and the like. Exemplary cyclic alkanes include, but are not limited to, cyclobutane (C<sub>4</sub>H<sub>8</sub>), cyclohexane (C<sub>6</sub>H<sub>12</sub>), and the like. Exemplary alkyl groups include, but are not limited to, methyl (-CH<sub>3</sub>), propyl (-C<sub>3</sub>H<sub>7</sub>), octyl (-C<sub>8</sub>H<sub>17</sub>), and the like. While alkanes are typically unreactive, alkyl groups can be combined with other

5 molecules to form compounds. For example, isopropyl alcohol (C<sub>3</sub>H<sub>7</sub>OH) is an alcohol that contains a propyl group. A general discussion of alkyl groups and alkanes can be found, among other places, in Morrison and Boyd, Organic Chemistry, 3<sup>rd</sup> Ed., Allyn and Bacon, Boston, MA, 1973; and Vollhardt, Organic Chemistry, W.H. Freeman, New York, NY, 1987.

10 The term "aryl group" refers to a hydrocarbon moiety that is based on benzene or other aromatic compounds. Aryl groups, like alkyl groups, can be combined with other molecules to form compounds. A general discussion of aromatic compounds and aryl groups can be found, among other places, in Morrison and Boyd, Organic Chemistry, 3<sup>rd</sup> Ed., Allyn and Bacon, Boston, MA, 1973; and Vollhardt, Organic Chemistry, W.H.

15 Freeman, New York, NY, 1987, particularly Chapter 19.

20 The term "biological sample" is used in a broad sense and is intended to include a variety of biological sources that contain nucleic acids. Such sources include, without limitation, whole tissues, including biopsy materials and aspirates; *in vitro* cultured cells, including primary and secondary cells, transformed cell lines, and tissue and cellular explants; whole blood, red blood cells, white blood cells, and lymph; body fluids such as urine, sputum, semen, secretions, eye washes and aspirates, lung washes and aspirates; and the like. Microorganisms and viruses that may be present on or in a biological sample are within the scope of the invention.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

5 The term "buffer," as used herein, refers to aqueous solutions or compositions that resist changes in pH when acids or bases are added to the solution. This resistance to pH change is due to the solution's buffering action. Solutions exhibiting buffering activity are referred to as buffers or buffer solutions. Buffers typically do not have an unlimited ability to maintain the pH of a solution or composition. Rather, typically they are able to maintain the pH within certain ranges, for example between pH 5 and pH 7. *See, generally*, C. Mohan, *Buffers*, A guide for the preparation and use of buffers in biological systems, Calbiochem, 1999. Exemplary buffers include, but are not limited to, MES ([2-(N-Morpholino)ethanesulfonic acid]), ADA (N-2-Acetamido-2-  
10 iminodiacetic acid), and Tris ([tris(Hydroxymethyl)aminomethane]; also known as Trizma); Bis-Tris; ACES; PIPES; MOPS; and the like (all available from Sigma).

15 Buffers that maintain the pH within a certain pH range, for example, between pH 5 and pH 7, and similar terms as used herein, are intended to encompass any buffer that exhibits buffering action at some point within the stated pH range. Thus, that term encompasses buffers that do not exhibit buffering capacity within the entire stated range, and buffers with buffering capacity that extend beyond the stated range. For example, solution A may exhibit buffering capacity between pH 5.2 and 6.7, solution B may exhibit buffering capacity between 6.0 and 8.0. For purposes of this invention, both of those solutions would be considered buffers that maintain the pH within the  
20 range of pH 5.0 to pH 7.0. The skilled artisan will be able to identify an appropriate buffer for maintaining the pH between a specified range using a buffer table. Buffer tables can be found in, among other places, the Calbiochem 2000-2001 General Catalog at pages 81-82, and the Sigma 2000-2001 Biochemicals and Reagents for Life

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Science Research Catalog at page 1873, both of which are expressly incorporated by reference.

5 The term "chaotrope" or "chaotropic salt," as used herein, refers to a substance that causes disorder in a protein or nucleic acid by, for example, but not limited to, altering the secondary, tertiary, or quaternary structure of a protein or a nucleic acid while leaving the primary structure intact. Exemplary chaotropes include, but are not limited to, guanidine hydrochloride, guanidinium thiocyanate, sodium thiocyanate, sodium iodide, sodium perchlorate, urea, and the like. A typical anionic chaotropic series, shown in order of decreasing chaotropic strength, includes:  $\text{CCl}_3\text{COO}^- \gg \text{CNS}^-$   
 10  $> \text{CF}_3\text{COO}^- > \text{ClO}_4^- > \text{I}^- > \text{CH}_3\text{COO}^- > \text{Br}^-, \text{Cl}^-, \text{or } \text{CHO}_2^-$ . Descriptions of chaotropes and chaotropic salts can be found, among other places, in Hamaguchi, Kozo and Geiduschek; E. Peter; Hatefi, Y. and Hanstein, W.G. (1962), "Solubilization of Particulate Proteins And Nonelectrolytes by Chaotropic Agents," Proc. Natl. Acad. Sci. 62: 1129-1136); The Effect Of Electrolytes On The Stability Of The Deoxyribonucleate  
 15 Helix, J. Amer. Chem. Soc. 84: 1329-1338); and U.S. Patent No. 5,234,809.

A "chelator" is a cyclic or heterocyclic ring compound that is capable of binding to metal cations, such as sodium ( $\text{Na}^+$ ), magnesium ( $\text{Mg}^{++}$ ), or calcium ( $\text{Ca}^{++}$ ) ions. At least two non-metal ions of the chelator form coordinate bonds with the metal cation to incorporate it into the ring. Thus, the metal cation is no longer free to participate in  
 20 other reactions. Cations that have a single charge, such as  $\text{Na}^+$  are referred to as monovalent cations, while those with a double charge, such as  $\text{Mg}^{++}$  or  $\text{Ca}^{++}$ , are referred to as divalent cations. Some enzymes, such as certain polymerases and nucleases typically function in the presence of free divalent cations. Thus, a divalent

LAW OFFICES

FINNEGAN, HENDERSON,  
 FARABOW, GARRETT  
 & DUNNER, L.L.P.  
 STANFORD RESEARCH PARK  
 700 HANSEN WAY  
 PALO ALTO, CALIF. 94304  
 650-849-6600

cation chelator, provided in sufficient amounts, typically would inhibit or reduce the activity of such divalent cation-dependent enzymes. Exemplary chelators include, but are not limited to, EDTA, EGTA, diaminoethane, and the like.

A "nuclease," such as a deoxyribonuclease (DNase) or a ribonuclease (RNase), is an enzyme that catalyzes the hydrolysis of phosphodiester linkages in nucleic acid polymers. Nucleases cause nucleic acid polymers to degrade, releasing constituent nucleotides and/or oligonucleotides (fragments of the polymers). A DNase degrades DNA molecules and an RNase degrades RNA molecules. Nuclease activity can typically be slowed or prevented by appropriate nuclease inhibitors.

As used herein, the term "organic solvent" refers to organic liquids, i.e., those comprising molecules with a hydrocarbon backbone. Organic solvents are capable of solvating non-solvent molecules, e.g., by surrounding them with solvent molecules so that the non-solvent molecules are dissolved in the solvent. Exemplary organic solvents include, but are not limited to, benzene, carbon tetrachloride, chloroform, phenol and other alcohols such as ethanol, methanol, and isopropanol (2-propanol), dimethyl sulfoxide (DMSO), and the like. Organic solvents can be used, for example, to extract nucleic acids from certain biochemical compositions, or to precipitate nucleic acids from aqueous solutions. Discussions of organic solvents can be found, among other places, in Morrison & Boyd, Organic Chemistry, 3d Edition, Allyn & Bacon, Boston, MA, 1973.

As used herein, the term "protease" refers to an enzyme that catalyzes the cleavage of peptide bonds, e.g., in proteins, polypeptides, oligopeptides, and peptides (collectively "peptides"). Exemplary proteases include, but are not limited to, subtilisins,

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

subtilases, alkaline serine proteases, and the like. Subtilases are a family of serine proteases, i.e., enzymes that cleave peptides after the amino acid serine. Subtilases are found in prokaryotic and eukaryotic organisms, such as bacteria, fungi, yeast, and higher organisms. Subtilisins are bacterial serine proteases that have broad substrate specificities. Subtilisins are relatively resistant to denaturation by chaotropes, such as urea and guanidine hydrochloride, and anionic detergents, such as sodium dodecyl sulfate. Exemplary subtilisins include, but are not limited to: Proteinase K; Proteinase R; Proteinase T (isolated from *Tritirachium album* Limber); Subtilisin DY, Carlsberg, also referred to as Subtilisin, Subtilisin A, Subtilopeptidase A, or Alcalase Novo; BPN', also referred to as Nagarse proteinase, Nagarse, or Subtilopeptidase C; Novo, also referred to as Bacterial proteinase Novo, Subtilisin B, or Subtilopeptidase B; mesentericopeptidase; Thermitase; and the like. Discussions of subtilases, subtilisins, Proteinase K, and other proteases may be found, among other places, in Genov et al., (1995) Stability Of Subtilisins And Related Proteinases (Subtilases), Int. J. Peptide Protein Res. 45: 391-400); Narhi and Arakawa (1989) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis As A Method For Studying The Stability Of Subtilisin, Biochimica et Biophysica Acta. Vol. 990: 144-149); Dixon and Webb, Enzymes, 3d Edition, Academic Press, New York, NY (1979); and Creighton, Proteins: Structures and Molecular Principles, W.H. Freeman and Co., New York, NY (1984).

The term "salt" as used herein, refers to a compound produced by the interaction of an acid and a base. Exemplary salts include, but are not limited to, sodium chloride (table salt), potassium phosphate, sodium bicarbonate, and the like. In water and other aqueous solutions, salts typically dissociate into an "anion" or negatively charged

subcomponent, and a "cation" or positively charge subcomponent. For example, when sodium chloride (NaCl) is dissolved in water, it dissociates into a sodium cation (Na<sup>+</sup>) and a chloride anion (Cl<sup>-</sup>).

As used herein, the term "surfactant" refers to a surface-active agent that generally comprises a hydrophobic portion and a hydrophilic portion. Examples of surfactants include, but are not limited to, detergents and bile acid salts. (See, e.g., Bhairi, A Guide to the Properties and Uses of Detergents in Biological Systems, Calbiochem-Novabiochem Corp. 1997). Surfactants may be categorized as anionic, nonionic, zwitterionic, or cationic, depending on whether they comprise one or more charged group. Anionic surfactants, such as SDS or lauryl sarkosine, contain a negatively charged group and have a net negative charge. Nonionic surfactants contain non-charged polar groups and have no charge. Exemplary nonionic surfactants include, but are not limited to, Tween 20, Triton X-100, NP-40, and the like. A zwitterionic surfactant contains both a positively charged group and a negatively charged group, and thus they are also uncharged.

A "cationic surfactant" has a positively charged group under the conditions examined. Cationic surfactants may contain quaternary amines or tertiary amines. Exemplary quaternary amine surfactants include, but are not limited to, cetylpyridinium chloride, cetyl trimethyl ammonium bromide (CTAB; Calbiochem #B22633 or Aldrich #85582-0), cetyl trimethyl ammonium chloride (CTACl; Aldrich #29273-7), dodecyl trimethyl ammonium bromide (Sigma #D-8638), octyl trimethyl ammonium bromide, tetradecyl trimethyl ammonium bromide, octadecyl trimethyl ammonium bromide, stearoalkonium chloride, olealkonium chloride, cetrimonium chloride, alkyl trimethyl

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600



ammonium methosulfate, palmitamidopropyl trimethyl chloride, quaternium 84 (Mackernium NLE; McIntyre Group, Ltd.), wheat lipid epoxide (Mackernium WLE; McIntyre Group, Ltd.), and the like. Exemplary ternary amine surfactants include, but are not limited to, octyldimethylamine, decyldimethylamine, dodecyldimethylamine, 5 tetradecyldimethylamine, hexadecyldimethylamine, octyldecyldimethylamine, octyldecylmethylamine, didecylmethylamine, dodecylmethylamine, triacetylammonium chloride, cetrimonium chloride, alkyl dimethyl benzyl ammonium chloride, and the like.

The term "whole tissue" according to the present invention is used in the broad sense to include any collection of cells organized to perform a specific function.

10 Examples of tissues include, without limitation: muscles, including cardiac, striated, and smooth muscle; organs, such as kidney, liver, spleen, brain, etc.; nerves; dermal and epidermal layers, such as skin; connective tissue such as bone, cartilage, ligaments, and tendons; and the like. Fragments, pieces, sections, slices, and sub-components of whole tissues are within the scope of the invention. The term whole tissue is not 15 limited, for example, to an entire organ or bone. Any aggregate or assembly of cells at any level is, for purposes of this invention, considered whole tissue. Plant tissues, such as leaves, roots, stems, and the like, are also within the scope of the present invention.

The term "nucleic acid," as used herein, refers to a polymer of ribonucleosides or deoxyribonucleosides comprising phosphodiester linkages between subunits. Such 20 nucleic acids include, but are not limited to, genomic DNA, hnRNA, mRNA, rRNA, tRNA, fragmented nucleic acid, nucleic acid obtained from subcellular organelles such as mitochondria or chloroplasts, and nucleic acid obtained from microorganisms or DNA or RNA viruses that may be present on or in a biological sample.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Solid phase components that are capable of binding to nucleic acids released from a biological sample include a variety of materials that are capable of binding nucleic acids under suitable conditions. Exemplary solid phase components include, but are not limited to, silica particles, silicon dioxide, diatomaceous earth, glass, alkylsilica, aluminum silicate, borosilicate, nitrocellulose, diazotized paper, hydroxyapatite, nylon, metal oxides, zirconia, alumina, diethylaminoethyl- and triethylaminoethyl-derivatized supports (Chromegabond SAX, LiChrosorb-AN, Nucleosil SB, Partisil SAX, RSL Anion, Vydac TP Anion, Zorbax SAX, Nucleosil NMe<sub>2</sub>, Aminex A-series, Chromex, and Hamilton HA Ionex SB, DEAE sepharose, QAE sepharose), hydrophobic chromatography resins (such phenyl- or octyl-sepharose), and the like.

Certain polymers, under appropriate conditions, are capable of precipitating nucleic acids from solution. Some polymers are capable of forming a nucleic acid-polymer complex that has a low solubility or is insoluble, so that complexed nucleic acid may precipitate. Examples of polymers that form such complexes with nucleic acids include, but are not limited to, polyethyleneimine, DEAE dextran, polylysine, polyarginine, polyhistidine, other weakly basic polymers (described in, among other places, U.S. Patent Nos. 5,582,988; 5,733,762; 5,622,822; and 5,599,667), and the like. Other polymers, such as polyethylene glycol, bind significant amounts of water in a solution, which may also cause nucleic acids to precipitate from the solution.

## Exemplary Embodiments of the Invention

The present invention is directed to compositions, methods, and kits for isolating nucleic acid from biological samples, preferably whole tissue. The compositions, methods, and kits of the invention reduce the time needed for sample preparation,

### LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

decrease potential safety risks posed by multi-step procedures that require repeated sample manipulation, and provide high integrity (i.e., minimally degraded) high molecular weight nucleic acid.

5 The compositions and kits comprise and the methods employ surfactants and proteases. The biological sample is combined with at least one cationic surfactant and at least one protease to form a reaction composition. The reaction composition is then incubated in a manner such that nucleic acids are released from the biological samples. For example, during incubation, these reaction compositions typically can: (i) macerate or disaggregate the biological sample; (ii) lyse the cells comprising the sample; (iii) 10 sterilize the sample; (iv) neutralize or inactivate nucleases; and (v) release nucleic acids from the biological sample.

While Proteinase K alone will effectively macerate whole tissue when incubated for approximately 12-18 hours, the released nucleic acid is frequently highly degraded. This degradation is due, at least in part, to nucleases present within the sample. The 15 maceration process can be accelerated, as disclosed herein, by the addition of a cationic surfactant to the reaction composition. Under appropriate conditions, in certain embodiments, high integrity nucleic acid can be efficiently obtained from biological samples in 60 minutes or less using the compositions and methods of the invention. Thus, the compositions and methods of the invention provide an unexpected 20 advantage.

Some cationic surfactants, under appropriate conditions, form a complex with the released nucleic acid and precipitate. These cationic surfactant:nucleic acid complexes

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

may be dissolved using a nonionic surfactant and an appropriate salt. Thus, in certain embodiments, the compositions further comprise a second surfactant and a salt.

In certain embodiments, the released nucleic acid is isolated. Nucleic acids can be isolated effectively from aqueous solutions that contain biomolecules, such as lipids, proteins, and the like, using organic solvents. For example, organic solvents such as phenol or combinations of phenol and chloroform can be used to extract the nucleic acids from such solutions. The extracted nucleic acids can be isolated by precipitating the nucleic acid using, for example, alcohols such as ethanol, propanol, or butanol. In other embodiments, the released nucleic acid is not isolated from the reaction composition.

Nucleic acids may also be isolated from the reaction composition using certain polymers or divalent cations for precipitating the nucleic acids. Exemplary polymers for precipitating nucleic acids include, but are not limited to, polyethylene glycol, polyethyleneimine, DEAE dextran, polylysine, polyarginine, polyhistidine, and other weakly basic polymers. Descriptions of such polymers may be found, among other places, in U.S. Patent Nos. 5,582,988; 5,733,762; 5,622,822; and 5,599,667. Certain divalent cations may also cause nucleic acid molecules to selectively precipitate from solution. Examples of such divalent cations include, but are not limited to, zinc chloride, magnesium chloride and magnesium sulfate.

Additionally, nucleic acids may be isolated using solid phase supports that selectively bind nucleic acids. For example, but not limited to, nucleic acids from a sample can be adsorbed to a solid phase in the presence of high concentrations of a chaotrope or salt. The solid phase may be washed to remove contaminating material,

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

and the nucleic acid can be eluted from the solid phase using a solution with a low ionic strength. Suitable solid phases include, but are not limited to, silica particles, silicon dioxide, diatomaceous earth, glass, alkylsilica, aluminum silicate, or borosilicate.

Also, nucleic acids can be bound to an ion exchange resin in the presence of low ionic strength. Following removal of the contaminating components by washing, the nucleic acid is eluted from the solid phase by increasing the ionic strength. Exemplary ion-exchange resins include, but are not limited to, diethylaminoethyl- and triethylaminoethyl-derivatized supports (Chromegabond SAX, LiChrosorb-AN, Nucleosil SB, Partisil SAX, RSL Anion, Vydac TP Anion, Zorbax SAX, Nucleosil NMe<sub>2</sub>, Aminex A-series, Chromex, and Hamilton HA Ionex SB, DEAE sepharose, or QAE sepharose, and the like). The skilled artisan will appreciate that other solid phase materials may also be used, for example, but not limited to, nitrocellulose, diazotized paper, hydroxyapatite, nylon, metal oxides, zirconia, alumina, and reverse-phase resins (such as octyl or phenyl sepharose).

The compositions of the invention include at least one protease. In certain embodiments, proteases such as subtilisins, subtilases, and alkaline serine proteases are employed. Exemplary proteases include, but are not limited to, Proteinase R, Proteinase T, subtilisin DY, dispase, subtilisin Carlsberg, subtilopeptidase A, thermolysin, thermostable proteases (such as those from *Thermus* Rt41A and *Bacillus thermoproteolyticus rokko*), and alkaline serine proteases from *Streptomyces griseus* or *Bacillus licheniformis*. A preferred protease, according to certain embodiments, is Proteinase K.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Certain proteases, including Proteinase K, are typically stabilized by the presence of calcium chloride. When calcium chloride is combined with certain anionic surfactants, such as SDS, a relatively insoluble precipitate can be formed. To avoid such precipitation, scientists sometimes omit calcium chloride when employing both protease and anionic surfactants. In preferred embodiments of the invention in which no substantial amount of anionic surfactants are employed, calcium chloride may be included.

To obtain high integrity DNA, in certain embodiments, DNase inhibitors may be added to the compositions of the invention. For example, many endogenous DNases are inhibited by divalent cation chelators, such as EDTA (ethylenediaminetetraacetic acid), EGTA ([ethyleneglycol-bis( -aminoethyl)-N,N,N',N'-tetraacetic acid]), DTPA (diethylenetriaminepentaacetic acid), and the like.

In certain embodiments, the at least one cationic surfactant comprises a quaternary amine, a tertiary amine, or both. In certain embodiments, the at least one cationic surfactant comprises cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACl), hexadecyltrimethylammonium bromide (HDTAB), or hexadecyltrimethylammonium chloride (HDTACl), and the at least one protease comprises Proteinase K.

In certain embodiments, isolating the nucleic acid comprises extracting the nucleic acid with an organic agent, such as, but without limitation, phenol, chloroform, or both phenol and chloroform. In certain embodiments, the nucleic acid may be precipitated with an organic agent, for example, without limitation, an alcohol such as isopropanol, ethanol, or butanol. A combination of extracting the nucleic acid with an

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

organic agent and precipitating the extracted nucleic acid with an organic agent is also within the scope of isolating the nucleic acid.

In certain embodiments, methods for isolating ribonucleic acid from a biological sample are provided comprising using a cationic surfactant, a protease, and a buffer. In certain embodiments, the cationic surfactant is CTAB (Sigma or Aldrich), CTACI (Sigma or Aldrich), HDTAB (Sigma #H-5882 or Fluka #52367), or HDTACI (Fluka #41199).

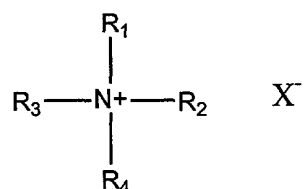
When high integrity RNA is desired, the presence of endogenous RNases sometimes limit the efficiency of some current procedures. Two components of the instant compositions and methods, however, can counteract the activity of endogenous RNases, enhancing the isolation of high integrity RNA. First, Proteinase K has been shown to inactivate RNase. Second, cationic surfactants may form complexes with RNA, decreasing the susceptibility of the RNA to RNase degradation. See, e.g., Dahl, C.E. and Macfarlane, D.E., Isolation Of RNA From Cells In Culture Using Catrimox-14™ Cationic Surfactant, BioTechniques Vol. 15, No. 6: 1102-1105 (1993); Macfarlane, D.E. and Dahle, C.E., Isolation RNA From Whole Blood -The Dawn Of RNA-Based Diagnosis? Nature, Vol. 362: 186-188 (1993); Macfarlane, D.E. and Dahle, C.E., Isolating RNA From Clinical Samples With Catrimox-14 and Lithium Chloride, Journal of Clinical Laboratory Analysis. 11: 132-139 (1997).

According to certain embodiments, endogenous RNase activity is further diminished using the compositions and methods of the invention. For example, RNases are less active at lower pH, e.g., between pH 5 and 7, and temperatures below 50° C.

Acridine orange can also decreases the nucleolytic activity of RNase, presumably by interacting with the released RNA molecules making them less susceptible to

degradation. RNase inhibitors may also be added to limit or prevent RNase activity. Exemplary RNase inhibitors include, but are not limited to, aurintricarboxylic acid, vanadylate ribonucleoside complexes, phenylglyoxal, p-hydroxyphenylglyoxal, polyamines, spermidine, 9-aminoacridine, iodoacetate, bentonite, poly[2'-O-(2,4-dinitrophenyl)]poly(adenylic acid), zinc sulfate, bromopyruvic acid, formamide, dimethylformamide, copper, zinc, and the like. In certain embodiments, the compositions and methods of the invention comprise at least one RNase inhibitor.

According to certain embodiments, cationic surfactants used in the compositions and methods have the general chemical formula:



( $N^+R_1R_2R_3R_4:X^-$ ), where the cation moieties  $R_1$ ,  $R_2$ ,  $R_3$ , and  $R_4$  independently may be: H, an alkyl group containing up to 20 carbon atoms, or an aryl group containing between 6 and 26 carbon atoms; wherein at least one of  $R_1$ ,  $R_2$ ,  $R_3$ , or  $R_4$  contains an alkyl group of at least 6 carbons; and where  $X^-$  is an anion. For example, the cationic surfactant may be an alkyltrimethyl ammonium salt, where  $R_1$ ,  $R_2$ , and  $R_3$  are methyl groups, and  $R_4$  is an alkyl group comprising 6, 8, 10, 12, 14, 16, or 18 carbon atoms. The cationic subcomponent ( $N^+R_1R_2R_3R_4$ ) of the alkyltrimethyl ammonium salt might be (without limitation) a cetyltrimethylammonium group, a hexadecyltrimethylammonium group, a tetradecyltrimethylammonium group, a dodecyltrimethylammonium group, a lauryl trimethylammonium group, or the like. The anionic subcomponent ( $X^-$ ) of this exemplary alkyltrimethyl ammonium salt might be (without limitation) any of the

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600



following ions: bromide, chloride, iodide, hydroxide, nitrate, sulfate, phosphate, formate, acetate, propionate, oxalate, malonate, succinate, or citrate. In certain embodiments, the cationic surfactant is a benzyldimethyl-n-alkylammonium salt, comprising the same group of anions.

5           The compositions and methods of the invention may further comprise solubilizing agents. Solubilizing agents are particularly useful when the biological sample is resistant to digestion, for example, when the sample comprises whole tissue. In certain embodiments, solubilizing agents help to disassemble the intercellular matrix, allowing the surfactant and protease to more effectively penetrate and disaggregate the sample.

10          Exemplary solubilizing agents include, but are not limited to, 1-methyl-2-pyrrolidinone, N-methyl pyrrolidinone, pyrrolidinone, dimethylsulfoxide, dimethylformamide, and the like.

          The invention also provides kits designed for releasing and/or for isolating nucleic acid from biological samples. The kits comprise at least one cationic surfactant and at least one protease, and according to certain embodiments may include additional

15          component(s). In certain embodiments, the kits preferably contain components in pre-measured unit amounts to minimize the measurements by end-users. In certain embodiments, the kits preferably include instructions for performing one or more methods of the invention. Preferably, the kit components are optimized to operate in conjunction with one another.

20           In certain embodiments, the at least one surfactant comprises CTAB, CTACl, HDTAB, HDTACl, or an equivalent. In certain embodiments, the at least one protease comprises Proteinase K. Optionally, other reagents for isolating extracted nucleic acid may be included in such kits. The kits of the invention may further comprise reaction

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

buffers, salts, ions, stabilizers, nuclease inhibitors, solubilizing agents, or combinations of these components.

The invention, according to certain embodiments, may be better understood by reference to examples. The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the invention in any way.

### **Example 1:**

An assay was developed to measure the activity of Proteinase K with BoDipy-labeled Casein Conjugate (Enz Check Protease Assay Kit , Product number E6638; Molecular Probes, Eugene, OR). The concentration of fluorescent BoDipy moieties in this derivatized conjugate is sufficiently high so that when the protein is intact, the fluorescence of individual moieties is quenched. Upon digestion of the derivatized casein by a protease, however, peptides containing a lower number of BoDipy dye molecules are released, resulting in an increase in fluorescence. To determine an initial dynamic range for an assay, BoDipy-labeled Casein was digested for one hour with a decreasing concentration of Proteinase K as follows.

Proteinase K (Product number 2546, Ambion) was added over a range of final concentrations (40 fg/ml to 40 µg/mL) to 500 µL of buffer (10 mM Tris, pH 8, 20 mM CaCl<sub>2</sub>) containing 10 µg/mL BoDipy-labeled Casein Conjugate. Following a one hour incubation at 60°C with mixing, 100 µL aliquots were transferred to a 96-well optical plate (MicroAmp® Optical 96-Well Reaction Plate, Applied Biosystems, Foster City, CA) and the fluorescence was measured in an ABI Prism 7700 spectrophotometer (Applied Biosystems). The background fluorescence was determined by incubating the substrate in buffer without Proteinase K.

#### **LAW OFFICES**

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

As shown in Figure 1, the amount of fluorescence increased as the Proteinase K concentration increased. The signal appeared to plateau at around 30,000 fluorescence units and released fluorescence was detected from reaction compositions containing as little as 50 ng/mL Proteinase K.

5

### Example 2:

10

Initially, a series of reaction compositions were analyzed for their ability to disaggregate a biological sample, e.g., slices of liver. These reaction compositions comprised 100 mM Tris, pH 8.0, 20 mM dithiothreitol (DTT), and optionally, 1 mg/mL Proteinase K, surfactants (e.g., cationic, nonionic and anionic detergents), chaotropes, or the additive 1-methyl-2-pyrrolidinone (Sigma). Each reaction compositions was placed in a tube with a slice of liver and then incubated at 65° C. The tubes were observed periodically to determine which reaction compositions were able to efficiently macerate the sample.

15

As shown in Table 1, Proteinase K alone was able to effectively digest the liver tissue, but only after extended incubation at 65° C. Of all of the reaction compositions examined, those containing the cationic surfactant CTAB most effectively macerated the sample during a one hour incubation period. Additionally, CTAB appeared to enhance the proteolytic activity of Proteinase K.

#### LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Table 1.

	65°C					
	No Proteinase K			1 mg/mL Proteinase K		
	2-5 hr	16-24 hr	72hr	1 hr	2-5 hr	16-24 hr
<b>Cationic Surfactants</b>						
Cetyltrimethylammonium bromide (1%)				100%		100%
Cetyltrimethylammonium bromide (0.1%)				100%		100%
<b>Nonionic Surfactants</b>						
Tween 80 (0.1%)				0%		0%
Tween 80 (1%)				0%		0%
Tween 80 (0.1%)				0%		
Triton X-100 (0.1%)				0%		0%
Triton X-100 (2%)	0%	0%				
Triton X-100 (1%)				30%		100%
<b>Anionic Surfactants</b>						
Chenodeoxycholic Acid (0.1%)				0%		0%
Chenodeoxycholic Acid (1%)				0%		0%
Chenodeoxycholic Acid (2%)		0%				
Cholic Acid (2%)		0%				
Glycocholic Acid (0.1%)				0%		0%
Glycocholic Acid (1%)				0%		0%
Taurochenodeoxycholic Acid (0.1%)				0%		0%
Taurochenodeoxycholic Acid (1%)				0%		0%
Taurocholic Acid (0.1%)				0%		0%
Taurocholic Acid (1%)				0%		0%
Taurocholic Acid (2%)		0%				
Taurodeoxycholic Acid (0.1%)				0%		0%
Taurodeoxycholic Acid (1%)				0%		0%
Octyl sulfonate (2%)			0%			
Hexane sulfonic acid (2%)			0%			
Octanonic Acid (2%)			0%			
SDS (1%)	0%				50%	
Lauryl Sarcosine (2%)			0%			
Saponin (0.1%)				0%		0%
Saponin (1%)				0%		0%
Saponin, (2%)	0%	50%				
NiaProof Type 8 (0.1%)				0%		0%
NiaProof Type 8 (1%)				0%		0%
Niaproof Type 8 (2%)	0%					
<b>Chaotropes</b>						
Guanidinium chloride (2M)	0%				0%	
Guanidinium thiocyanate (1M)	0%				25%	
Tetramethylammonium chloride (0.1M)		0%		0%		0%
Tetramethylammonium chloride (1M)		0%		0%		0%
<b>Other additives</b>						
1-methyl 2-pyrrolidinone (2%)		0%				
<b>No Additives</b>						
None					0%	75%

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

**Example 3:**

To test the effect of cationic surfactants on the activity of Proteinase K, a series of reaction compositions were prepared as follows. Reaction compositions comprised: Casein BoDipy conjugate (1  $\mu\text{g}$ , 10  $\text{ng}/\mu\text{L}$ ), 10 mM Tris, pH 8, 20 mM  $\text{CaCl}_2$ , 1% surfactant (shown in Table 2 below) and a dilution series of Proteinase K at concentrations of 20  $\mu\text{g}/\text{mL}$ , 5  $\mu\text{g}/\text{mL}$ , 1.25  $\mu\text{g}/\text{mL}$ , 0.31  $\mu\text{g}/\text{mL}$ , 0.078  $\mu\text{g}/\text{mL}$ , 0.02  $\mu\text{g}/\text{mL}$  and 0.005  $\mu\text{g}/\text{mL}$ . The anionic surfactant sodium dodecyl sulfate (SDS), known to activate Proteinase K activity under the conditions tested, was included as a positive control. Reaction tubes were incubated at 60°C in a 96-well optical plate (MicroAmp® Optical 96-Well Reaction Plate, Applied Biosystems) and the amount of released fluorescence was measured at the indicated time points using an ABI Prism 7700.

Table 2. Surfactants

Surfactant	Source
Olealkonium chloride	(CAS No.: 37139-99-4, McIntyre Group LTD).
Mackernium SDC-85 (Stearalkonium chloride)	(CAS No. 122-19-0, McIntyre Group LTD).
Mackernium NLE	(Quaternium-84)(McIntyre Group LTD)
Mackernium 006	(Polyquaternium-6) (Cas No. 26062-79-3) (McIntyre Group, LTD)
Mackernium 007	(Polyquaternium-7) (Cas No. 26590-05-6) (McIntyre Group, LTD)
Mackernium WLE	(Wheat Germamidopropyl Epoxy Q) (McIntyre Group, LTD)
Benzalkonium Chloride	(Sigma, Product Number B-6295)
Benzyltrimethylhexadecylammonium Chloride	(Sigma, Product Number B-4136)
Benzyltrimethyltetradecylammonium Chloride	(Sigma, Product Number B-5651)
Benzyltrimethyldodecylammonium bromide	(Sigma, Product Number B-5776)
Tetradecyltrimethylammonium Bromide	(Sigma, Product Number T-4762)
Hexadecyltrimethylammonium Bromide	(Sigma, Product Number H-5882)
Dodecyltrimethylammonium Bromide	(Sigma, Product Number D-8638)
Mixed Alkyltrimethylammonium Bromide	(Sigma, Product Number M-7635)
Cetyltrimethylammonium bromide (CTAB)	(Calbiochem, Product number B22633)
Sodium Dodecyl Sulfate (SDS)	(Sigma, Product Number L6026)

As shown in Figures 2A-C: (i) Proteinase K without surfactant has high proteolytic activity, even at ambient temperature (see 0 minutes incubation in water); (ii)

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK

700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Calf thymus DNA (Sigma; 700 µg/mL lightly sheared using an 18 gauge hypodermic needle) was mixed with 1% CTAB in 100 mM Tris, pH 8 to form a reaction composition. Initially, a noticeable precipitate was observed. A variety of additives, shown in Table 3, were tested with aliquots of this reaction composition to identify compositions that would solubilize the cationic surfactant:nucleic acid precipitate.

5

1000 900 800 700 600 500 400 300 200 100 0

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Table 3.

Sample	Additive (final concentration)	Result
1	91 mM NaCl	Nucleic acid precipitated
2	333 mM NaCl	Nucleic acid precipitated
3	117 mM Tetramethylammonium chloride	Nucleic acid precipitated
4	758 mM Tetramethylammonium chloride	Nucleic acid precipitated
5	95 mM Tetrabutylammonium chloride	Nucleic acid precipitated
6	400 mM Tetrabutylammonium chloride	Nucleic acid precipitated
7	312.5 mM NaCl, 6.25% 1-Methyl-2-Pyrrolidinone	Nucleic acid precipitated
8	692 mM Tetramethylammonium chloride, 7.5% 1-Methyl-2-pyrrolidinone	Nucleic acid precipitated
9	370 mM Tetrabutylammonium chloride, 7.4% 1-Methyl-2-pyrrolidinone	Nucleic acid precipitated
10	9.1% 1-Methyl-2-pyrrolidinone (Sigma #M-6762)	Nucleic acid precipitated
11	294 mM NaCl, 5.9% 1-Methyl-2-Pyrrolidinone, 5.9% Tween 20	Nucleic acid pellet fairly dissolved
12	643 mM Tetramethylammonium chloride, 7.0% 1-Methyl-2-pyrrolidinone, 7.0% Tween 20	Nucleic acid pellet fairly dissolved
13	345 mM Tetrabutylammonium chloride, 6.9% 1-Methyl-2-Pyrrolidinone, 6.9% Tween 20	Nucleic acid pellet began to solubilize
14	8.3% 1-Methyl-2-Pyrrolidinone, 8.3% Tween 20	Nucleic acid pellet began to solubilize
15	10% Tween 20 (Sigma #P-9416)	Nucleic acid precipitated
16	312.5 mM NaCl, 6.25% Tween 20	Nucleic acid pellet completely solubilized
17	2.0% Tween 20	Nucleic acid precipitated
18	160 mM NaCl, 1.6% Tween 20	Nucleic acid precipitated
19	9.1% 1-hexanesulfonic acid	Nucleic acid pellet partially solubilized
20	17% 1-hexanesulfonic acid	Nucleic acid pellet partially solubilized
21	200 mM NaCl, 16% Hexane sulfonic acid	Nucleic acid pellet partially solubilized
22	9.1% Decanesulfonic acid	Nucleic acid pellet partially solubilized
23	217 mM NaCl, 8.7% Decanesulfonic acid	Nucleic acid pellet partially solubilized
24	50% Nucleic Acid Purification Lysis Solution	Nucleic acid pellet completely solubilized
25	33% Nucleic Acid Purification Lysis Solution	Nucleic acid pellet completely solubilized
26	23% Nucleic Acid Purification Lysis Solution	Nucleic acid pellet partially solubilized
27	297 mM NaOAc	Nucleic Acid precipitated
28	292 mM NaOAc, 1.8% Tween 20	Nucleic Acid precipitated
29	313 mM NaOAc, 2.6% Tween 20	Nucleic Acid precipitated
30	535 mM NaCl, 1.75% Tween 20	Nucleic acid pellet completely solubilized

As shown in Table 3, four of the reaction compositions resulted in complete solubilization of the precipitate. Nucleic Acid Purification Lysis Solution (# 4305895, Applied Biosystems), used at final concentrations of 33% or 50%, completely solubilized

## LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

the precipitate (see samples 24-25). Additionally, some combinations of the salt NaCl and the nonionic detergent Tween 20 completely solubilized the precipitate (see samples 16 and 30). However, neither NaCl nor Tween 20, used alone, solubilized the precipitate (see samples 1, 2, 15, and 17). The skilled artisan will understand that additional combinations of salts and nonionic surfactants used at varying concentrations can be used to dissolve the cationic surfactant:nucleic acid precipitates.

### Example 5:

To evaluate the ability of various cationic surfactants to increase the digestion of tissue by Proteinase K, liver slices were digested using reaction compositions including one of the sixteen surfactants (shown in Table 4), both with and without Proteinase K. The reducing agent dithiothreitol (DTT) was included to evaluate the effect of reducing conditions on tissue digestion. Liver tissue (80 - 120 mg) was placed into each of thirty-four Eppendorf tubes (seventeen pairs of tubes) containing 1000  $\mu$ L of 10 mM Tris, pH 8, 20 mM  $\text{CaCl}_2$ , and 20 mM dithiothreitol. One of the sixteen surfactants was added to one pair of tubes to yield a final concentration of 1% surfactant. The seventeenth pair of tubes served as non-surfactant controls. One milligram (mg) of Proteinase K was added to one tube of each of the seventeen pairs. The thirty-four tubes were then incubated at 60°C with mixing (Eppendorf Thermomixer, Model 5436).

#### LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600



Table 4.

Surfactant	Chemical Formula
SDS	$\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na}$
Mackernium KP	$\text{R}_1\text{R}_2(\text{CH}_3)_2\text{NCl}$ $\text{R}_1$ =fatty group, $\text{R}_2$ =benzyl, methyl or epoxy group
Mackernium WLE	$\text{R}_1\text{R}_2(\text{CH}_3)_2\text{NCl}$ $\text{R}_1$ =fatty group, $\text{R}_2$ =benzyl, methyl or epoxy group
Benzalkonium chloride	$\text{C}_6\text{H}_5\text{CH}_2\text{N}(\text{CH}_3)_2\text{RCl}$ $\text{R}=\text{C}_8\text{H}_{17}$ to $\text{C}_{18}\text{H}_{37}$
Mackernium 006	Polyquaternium
Mackernium 007	Polyquaternium
Mackernium NLE	$\text{R}_1\text{R}_2(\text{CH}_3)_2\text{NCl}$ $\text{R}_1$ =fatty group, $\text{R}_2$ =benzyl, methyl or epoxy group
Mackernium SDC-85	$\text{R}_1\text{R}_2(\text{CH}_3)_2\text{NCl}$ $\text{R}_1$ =fatty group, $\text{R}_2$ =benzyl, methyl or epoxy group
Dodecyltrimethylammonium bromide	$\text{CH}_3(\text{CH}_2)_{11}\text{N}(\text{CH}_3)_3\text{Br}$
Tetradecyltrimethylammonium bromide	$\text{CH}_3(\text{CH}_2)_{13}\text{N}(\text{CH}_3)_3\text{Br}$
Hexadecyltrimethylammonium bromide	$\text{CH}_3(\text{CH}_2)_{15}\text{N}(\text{CH}_3)_3\text{Br}$
CTAB	$\text{CH}_3(\text{CH}_2)_{15}\text{N}(\text{CH}_3)_3\text{Br}$
Mixed alkyltrimethylammonium bromide	$\text{CH}_3(\text{CH}_2)_n\text{N}(\text{CH}_3)_3\text{Br}$
Benzyltrimethylammonium bromide	$\text{C}_6\text{H}_5\text{CH}_2\text{N}[(\text{CH}_2)_{11}\text{CH}_3](\text{CH}_3)_2\text{Br}$
Benzyltrimethyltetradecyl ammonium chloride	$\text{C}_6\text{H}_5\text{CH}_2\text{N}[(\text{CH}_2)_{13}\text{CH}_3](\text{CH}_3)_2\text{Cl}$
Benzyltrimethylhexadecylammonium bromide	$\text{C}_6\text{H}_5\text{CH}_2\text{N}[(\text{CH}_2)_{15}\text{CH}_3](\text{CH}_3)_2\text{Br}$

Each tube was visually evaluated after 15 minutes and 52 minutes of incubation.

The results, shown in Table 5 (fifteen minutes incubation) and Table 6 (fifty-two minutes incubation), indicate that under the conditions tested, the combination of CTAB and Proteinase K caused total disaggregation of the tissue sample at either time point. Notably, CTAB alone caused no apparent sample disaggregation. The presence of DTT did not seem to significantly effect tissue disaggregation under these conditions.

## LAW OFFICES

FINNEGAN, HENDERSON,  
 FARABOW, GARRETT  
 & DUNNER, L.L.P.  
 STANFORD RESEARCH PARK  
 700 HANSEN WAY  
 PALO ALTO, CALIF. 94304  
 650-849-6600

Table 5. Observations after Fifteen Minutes Incubation.

Surfactant	PK	Tissue Disaggregation	Color of Composition
Buffer alone (control)	Y	0	Light pink
SDS	Y	3	Slightly Cloudy
Mackernium KP	Y	1	Slightly Cloudy
Mackernium WLE	Y	0	Clear
Benzalkonium chloride	Y	1	Slightly Cloudy
Mackernium 006	Y	1	Slightly Cloudy
Mackernium 007	Y	2	Slightly Cloudy
Mackernium NLE	Y	1	Slightly Cloudy
Dodecyltrimethylammonium bromide	Y	2	Medium Cloudy
Tetradecyltrimethylammonium bromide	Y	0	Clear
Hexadecyltrimethylammonium bromide	Y	0	Clear
CTAB	Y	4	Dark brown
Mackernium SDC-85	Y	3	Medium cloudy
Mixed alkyltrimethylammonium bromide	Y	3	Medium cloudy
Benzyltrimethylhexadecylammonium bromide	Y	0	Clear
Benzyltrimethylhexadecylammonium bromide	Y	0	Clear
Benzyltrimethyltetradecyl ammonium chloride	Y	0	Clear
Buffer alone (control)	N	0	Pink
SDS	N	0	Slightly cloudy
Mackernium KP	N	0	Clear
Mackernium WLE	N	0	Pink
Benzalkonium chloride	N	0	Clear
Mackernium 006	N	0	Slightly pink
Mackernium 007	N	0	Slightly pink
Mackernium NLE	N	0	Slightly brown
Dodecyltrimethylammonium bromide	N	0	Slightly pink
Tetradecyltrimethylammonium bromide	N	0	Slightly pink
Hexadecyltrimethylammonium bromide	N	0	Clear
CTAB	N	0	Slightly pink
Mackernium SDC-85	N	0	Clear
Mixed alkyltrimethylammonium bromide	N	0	Clear
Benzyltrimethylhexadecylammonium bromide	N	0	Slightly cloudy
Benzyltrimethylhexadecylammonium bromide	N	0	Clear
Benzyltrimethyltetradecyl ammonium chloride	N	0	Clear

PK= presence or absence of Proteinase K in the reaction composition; Y= Proteinase K was present; N= Proteinase K was not present in the reaction composition. Tissue disaggregation: 0 = none; 1 = No fragmentation, supernatant slightly cloudy; 2 = Little fragmentation, supernatant slightly cloudy; 3 = More significant tissue supernatant, supernatant very cloudy; 4 = complete tissue aggregation with little insoluble material remaining.

5

## LAW OFFICES

FINNEGAN, HENDERSON,  
 FARABOW, GARRETT  
 & DUNNER, L. L. P.  
 STANFORD RESEARCH PARK  
 700 HANSEN WAY  
 PALO ALTO, CALIF. 94304  
 650-849-6600

Table 6. Observations after Fifty-Two Minutes Incubation.

Surfactant	PK	Tissue Disaggregation	Color of Composition
Buffer alone (control)	Y	0	Slightly brown
SDS	Y	3	Moderately cloudy
Mackernium KP	Y	4	Very cloudy
Mackernium WLE	Y	2	Medium cloudy
Benzalkonium chloride	Y	2	Medium cloudy
Mackernium 006	Y	0	Slightly brown
Mackernium 007	Y	2	Some cloudiness
Mackernium NLE	Y	1	Slightly cloudy
Dodecyltrimethylammonium bromide	Y	3	Medium cloudy
Tetradecyltrimethylammonium bromide	Y	1	Slightly brown
Hexadecyltrimethylammonium bromide	Y	1	Medium brown
CTAB	Y	4	Very brown, milky
Mackernium SDC-85	Y	3	Very cloudy
Mixed alkyltrimethylammonium bromide	Y	3	Very cloudy
Benzyltrimethylammonium bromide	Y	1	Slightly cloudy
Benzyltrimethylhexadecylammonium bromide	Y	1	Slightly cloudy
Benzyltrimethyltetradecyl ammonium chloride	Y	1	Slightly cloudy
Water	N	0	Slightly brown
SDS	N	0	Slightly pink
Mackernium KP	N	0	Clear
Mackernium WLE	N	0	Slightly brown
Benzalkonium chloride	N	0	Clear
Mackernium 006	N	0	Slightly brown
Mackernium 007	N	0	Slightly cloudy
Mackernium NLE	N	0	Clear
Dodecyltrimethylammonium bromide	N	0	Clear
Tetradecyltrimethylammonium bromide	N	0	Clear
Hexadecyltrimethylammonium bromide	N	0	Slightly brown
CTAB	N	0	Slightly brown
Mackernium SDC-85	N	0	Slightly pink
Mixed alkyltrimethylammonium bromide	N	0	Clear
Benzyltrimethylammonium bromide	N	0	Slightly brown
Benzyltrimethylhexadecylammonium bromide	N	0	Clear
Benzyltrimethyltetradecyl ammonium chloride	N	0	Clear

See legend to Table 5 for explanation of nomenclature.

## LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

**Example 6:**

To examine the ability of cationic surfactants and Proteinase K to release high integrity nucleic acids from a biological sample, the released nucleic acid was isolated. Slices of liver tissue (90 – 200 mg per slice) were placed into each of twenty-two Eppendorf microfuge tubes. Four hundred  $\mu\text{L}$  of 50 mM Tris, pH 8, 20 mM  $\text{CaCl}_2$ , 1% surfactant, and 1 mg Proteinase K was added to each tube. The tubes were incubated at  $60^\circ\text{C}$  for 22 minutes with mixing (Eppendorf Thermomixer, Model 5436).

Following the incubation, Nucleic Acid Purification Lysis Solution (# 4305895, Applied Biosystems; final concentration 29%), sodium acetate (final concentration 214 mM) and glycogen (Mussel type VII, Sigma; final concentration  $143 \mu\text{g/mL}$ ) was added to each tube to dissolve the surfactant:nucleic acid complexes. The tubes were centrifuged in an Eppendorf microfuge (Model 5415C) at 14,000 RPM for 5 minutes to pellet remaining tissue fragments. The resulting supernatant was extracted using 700  $\mu\text{L}$  of phenol (Sigma) saturated with 10 mM Tris HCl, pH 8.0, and 1 mM EDTA (saturated phenol). The pelleted material was also extracted with saturated phenol to obtain nucleic acid if any remained in the undigested sample. The extracted nucleic acid was precipitated using an equal volume (750  $\mu\text{L}$ ) of 2-propanol, and the tubes were incubated at  $-20^\circ\text{C}$  for 3 hours. The samples were centrifuged at ambient temperature in a microfuge at 14,000 RPM for 5 minutes, as before, to pellet the nucleic acid. The nucleic acid pellets were washed with 1 mL of 70% ethanol and the samples were centrifuged at ambient temperature in a microfuge at 14,000 RPM for 5 minutes. The washed nucleic acid pellets were resuspended in 100  $\mu\text{L}$  10 mM Tris, pH 8, 0.1 mM EDTA, and 1 unit/ $\mu\text{L}$  RNasin. The amount of released nucleic acid was quantitated

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

using a UV/Vis Spectrophotometer (Hewlett Packard, Model 8453). For quantitation purposes, it was assumed that the material that absorbed UV light at a wavelength of 260 nm was nucleic acid. Thus, an extinction coefficient of  $1 \text{ OD}_{260} = 40 \mu\text{g/mL}$  was used.

5           Figure 3 shows the amount of  $A_{260}$ -absorbing material recovered from the sample ( $\mu\text{g}$  nucleic acid/mg tissue processed). The results were normalized for the weight of each sample. Despite the reduced proteolytic activity of Proteinase K in the presence of the cationic surfactants, as demonstrated in the previous example, the amount of  $A_{260}$ -absorbing material was similar to the amount obtained with the anionic surfactants or  
10           without surfactant.

          To evaluate the integrity of the released nucleic acid (i.e., its degree of degradation), and the amount of nucleic acid remaining in the tissue pellet, one tenth of each sample was analyzed by electrophoresis on a 1% agarose gel according to standard molecular biology procedures. Descriptions of standard molecular biology  
15           procedures may be found, among other places, in Molecular Cloning: A Laboratory Manual, Sambrook et al., 1989, Cold Spring Harbor Press, Cold Spring Harbor, NY; Current Protocols in Molecular Biology, Ausbel et al., 1993 and supplements through September 2000, John Wiley & Sons, New York, NY; or Molecular Biology Techniques, W. Ream et al., Academic Press, San Diego, CA, 1999.

20           As shown in Figure 4, most of the cationic surfactant:Proteinase K combinations released nucleic acid as evidenced by the little nucleic acid that remained in the pellet. The released nucleic acid was observed to have a high electrophoretic mobility, since it appeared near the bottom of the gel. Since intact, high integrity nucleic acid typically

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

has a low, rather than a high, electrophoretic mobility, the recovered nucleic acid apparently was highly fragmented using these conditions. In other words, it apparently had a low integrity.

**Example 7:**

5           The results from Example 6 indicate that there was significant nuclease activity present in the sample. To reduce the nucleolytic activity of endogenous RNAses, the pH of the reaction composition was lowered to approximately 6.0 and the incubation temperature was decreased to 45° C. Several additives, including phenylglyoxal (Aldrich #14243-3), 1-methyl-2-pyrrolidinone, RNA Later (Ambion, #7020), and Streck

10   Tissue Fixative (Streck Laboratories, # 265138) were also tested to evaluate their effect on endogenous RNase activity in these reaction compositions. Phenylglyoxal is known to reduce ribonuclease activity (see Takahashi, The Structure And Function Of Ribonuclease T<sub>1</sub>. XI. Modification Of The Single Arginine Residue In Ribonuclease T<sub>1</sub> By Phenylglyoxal And Glyoxal, J. Biochem (Japan) 68:659-664, 1970). The product

15   literature for RNA Later claims that the reagent stabilizes RNA in tissue and Streck Tissue Fixative is believed to preserve the integrity of tissue.

          Slices of liver tissue (100 - 200 mg/sample) were placed into nineteen Eppendorf tubes. Four hundred microliters of a reaction composition comprising 100 mM MES (Sigma #M-5287), pH 6, 20 mM CaCl<sub>2</sub>, and 1 mg Proteinase K, was added to each

20   tube. Additional components, including anionic surfactants (SDS, Sarkosyl) and cationic surfactants (CTAB, CTACl, and tetramethyl ammonium chloride) were added to specific tubes, as shown in Table 7.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Table 7.

Tube #	Additional Reaction Composition Components
1	No additional components (control)
2	1% Sodium Dodecyl Sulfate (SDS)
3	1% SDS, 10% 1-Methyl-2-pyrrolidinone
4	1% SDS, 100 mM Phenylglyoxal
5	1% SDS, 80% RNA Later
6	1% SDS, 80% Streck Tissue Fixative
7	1% Cetyltrimethylammonium bromide (CTAB)
8	1% CTAB, 10% 1-Methyl-2-pyrrolidinone
9	1% CTAB, 100 mM Phenylglyoxal
10	1% CTAB, 80% RNA Later
11	1% CTAB, 80% Streck Tissue Fixative
12	1% Sarkosyl
13	1% Sarkosyl, 10% 1-Methyl-2-pyrrolidinone
14	1% Sarkosyl, 10 mM Phenylglyoxal
15	1% Sarkosyl, 80% RNA Later
16	1% Sarkosyl, 80% Streck Tissue Fixative
17	1% Cetyltrimethylammonium chloride (CTACl)
18	600 mM Tetrabutylammonium chloride
19	800 mM Tetramethylammonium chloride

The tubes were incubated at 45°C for 20 minutes with mixing (Eppendorf Thermomixer, Model 5436). Following the incubation, 171  $\mu$ L of a solution containing 1.75 M NaCl, 29% Tween 20, 585  $\mu$ g/mL glycogen (447 mM NaCl, 7.5% Tween 20, and 149  $\mu$ g/mL glycogen) was added to each tube to dissolve the surfactant:nucleic acid complex. Glycogen served as a nucleic acid carrier. Undigested tissue fragments were removed by centrifugation using the methods the same as or similar to those discussed in Example 6. The supernatant was phenol extracted (saturated with 10 mM Tris HCl, pH 8, 1 mM EDTA; Sigma # P-4557), isopropanol precipitated (Aldrich #19076-4), and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspend with 100  $\mu$ L 10 mM Tris, pH 8.0, 0.1 mM EDTA, and 1 unit/ $\mu$ L Rnasin. The amount of nucleic acid released and its integrity were evaluated as described in Example 6.

LAW OFFICES

FINNEGAN, HENDERSON,  
 FARABOW, GARRETT  
 & DUNNER, L.L.P.  
 STANFORD RESEARCH PARK  
 700 HANSEN WAY  
 PALO ALTO, CALIF. 94304  
 650-849-6600

As shown in Figure 5, under these conditions, Proteinase K combined with either of the cationic surfactants (CTAB and CTACl) or the anionic surfactants (SDS and Sarkosyl) released  $A_{260}$  absorbing material from the sample. When either Streck Tissue Fixative or RNA Later was added to these reactions, very little  $A_{260}$  absorbing material was released. The average molecular weight for the released nucleic acid was typically higher when Proteinase K digestion was carried out at the lower pH and temperatures (compare Figure 4 to Figure 6). Additionally, the average molecular weight for the released nucleic acid was typically higher when Proteinase K digestion was performed in the presence of the cationic surfactants (compare Lanes 5-9 and 15-19 to 10-14 in Figure 6).

#### Example 8:

To compare CTACl to CTAB, those two surfactants were evaluated in parallel along with hexadecyltrimethylammonium bromide (HDTAB), hexadecyltrimethylammonium chloride (HDTACl), and Sarkosyl. Three additives, phenylglyoxal, acridine orange (e.g., Sigma #A6014), and 1-methyl-2-pyrrolidinone, were also evaluated. Phenylglyoxal was tested to determine if it would reduce RNase activity. Acridine Orange, a dye compound known to interact with nucleic acid molecules, was tested to determine if it would reduce nuclease activity. 1-Methyl-2-pyrrolidinone was tested to see if it would enhance solubility of the sample by various reaction compositions.

Slices of liver tissue (100 - 200 mg/sample) were placed into twenty-one Eppendorf tubes. Four hundred microliters of a reaction composition, comprising 100

LAW OFFICES

FINNEGAN, HENDERSON,  
 FARABOW, GARRETT  
 & DUNNER, L.L.P.  
 STANFORD RESEARCH PARK  
 700 HANSEN WAY  
 PALO ALTO, CALIF. 94304  
 650-849-6600



mM MES, pH 6, 20 mM CaCl<sub>2</sub>, and 1 mg Proteinase K, was added to each tube.

Additional components were added to specific tubes, as shown in Table 8.

Table 8.

Tube #	Additional Reaction Composition Components
1	1% Cetyltrimethylammonium Bromide (CTAB)
2	1% CTAB, 100 mM Phenylglyoxal
3	1% CTAB, 10 % 1-Methyl-2-pyrrolidinone
4	1% CTAB, 200 µg/mL Acridine orange
5	1% Cetyltrimethylammonium Chloride (CTACI)
6	1% CTACI, 100 mM Phenylglyoxal
7	1% CTACI, 10% 1-Methyl-2-pyrrolidinone
8	1% CTACI, 200 µg/mL Acridine orange
9	1% Hexatrimethylammonium Bromide (HTMAB)
10	1% HTMAB, 100 mM Phenylglyoxal
11	1% HTMAB, 10% 1-Methyl-2-pyrrolidinone
12	1% HTMAB, 200 µg/mL Acridine orange
13	1% Hexatrimethylammonium Chloride (HTMACI)
14	1% HTMACI, 100 mM Phenylglyoxal
15	1% HTMACI, 10% 1-Methyl-2-pyrrolidinone
16	1% HTMACI, 200 µg/mL Acridine orange
17	1% Sarkosyl (N-lauryl sarcosine)
18	1% Sarkosyl, 100 mM Phenylglyoxal
19	1% Sarkosyl, 10% 1-Methyl-2-pyrrolidinone
20	1% Sarkosyl, 200 µg/mL Acridine orange
21	No additives

5 The tubes were incubated at 45°C for 30 minutes with mixing (Eppendorf Thermomixer, Model 5436). Surfactant:protease complexes were solubilized using methods the same as or similar to those described in Example 7. Remaining tissue fragments were removed by centrifugation using methods the same as or similar to those described in Example 7. The supernatant was phenol extracted, isopropanol

10 precipitated, and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspended and the amount and the integrity of the released nucleic acid were evaluated using methods the same as or similar to those described in Example 6.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

As shown in Figure 7, the effect of the three additives varied depending on the surfactant. For example, the amount of nucleic acid released using CTACI and 1-methyl-2-pyrrolidinone was greater in comparison with a composition of CTACI without an additive. However, the amount of nucleic acid released using HDTAB and 1-methyl-2-pyrrolidinone was less in comparison with a composition of HDTAB without an additive.

When the released nucleic acid was analyzed by gel electrophoresis, high molecular weight nucleic acid was apparent in all reaction compositions containing HDTAB (see Figure 8). High molecular weight nucleic acid was also present in reaction compositions containing CTACI and either 1-methyl-2-pyrrolidinone or acridine orange. High molecular weight nucleic acid was also observed in the reaction composition comprising HDTACI and 1-methyl-2-pyrrolidinone.

#### **Example 9:**

Example 7 demonstrated that the presence of certain reagents that have been used to stabilize RNA in tissue samples (*i.e.* Streck Tissue Fixative and RNA Later) inhibited the activity of Proteinase K. To attempt to reduce this inhibition, tissue was pre-incubated in several reagents to allow them to diffuse into the sample. Residual reagent was removed prior to incubation of the samples in reaction compositions.

Liver slices were incubated in various solutions prior to exposure to reaction compositions as follows. Slices of liver tissue (94 – 330 mg/sample) were placed into four sets of six Eppendorf tubes. Five hundred microliters of either Nucleic Acid Purification Lysis Solution, RNA Later, or Streck Tissue Fixative was added to one set

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L. L. P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

of tubes. The fourth set of tubes contained no pretreatment solution and served as a control.

The tubes were incubated for 4 hours at 4°C, then centrifuged in an Eppendorf microfuge (Model 5415C) at ambient temperature for 2 minutes at 14,000 RPM. The supernatant was discarded and four hundred microliters of a reaction composition comprising 100 mM MES, pH 6.0, 20 mM CaCl<sub>2</sub>, and 1 mg Proteinase K, was added to each tube. One of the six additional components shown in Table 9 was also added to each tube. The tubes were incubated at 45°C with mixing (Eppendorf Thermomixer, Model 5436) for 30 minutes.

The resulting surfactant:protease complex was solubilized using methods the same as or similar to those described in Example 7. Remaining tissue fragments were removed by centrifugation using methods the same as or similar to those described in Example 7. The supernatant was phenol extracted, isopropanol precipitated, and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspended and the amount of nucleic acid released and the integrity of that nucleic acid were evaluated using methods the same as or similar to those described in Example 6.

Table 9.

Tube	Additional Reaction Composition Components
1	1% Cetyltrimethylammonium Bromide (CTAB)
2	1% CTAB, 10% 1-methyl-2-pyrrolidinone (NMP)
3	1% CTAB, 200 µg/mL Acridine Orange
4	1% Cetyltrimethylammonium Chloride (CTACl)
5	1% CTACl, 10% NMP
6	1% CTACl, 200 µg/mL Acridine Orange

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

As shown in Figure 9, pretreatment of the samples with one of the pretreatment solutions resulted in a decreased amount of A<sub>260</sub>-absorbing material that was released when compared to the control.

### Example 10:

5 The ability of aurintricarboxylic acid to maintain the integrity of the nucleic acid released using reaction compositions comprising CTAB, CTACI, or SDS, was evaluated. Aurintricarboxylic acid is believed to inhibit the binding of proteins, e.g., nucleases, to nucleic acid and has been demonstrated to inhibit ribonucleases. Slices of liver tissue (77 - 186 mg/sample) were placed into twenty-one Eppendorf tubes. To  
10 each tube was added 400  $\mu$ L of a reaction composition comprising 50 mM MES, pH 6.0, 20 mM CaCl<sub>2</sub>, and additional components as shown in Table 10.

Table 10. Additional Reaction Composition Components

Tube	Additional Reaction Composition Components
1	1% CTAB, 5 mM Aurintricarboxylic Acid
2	1% CTAB, 2 mM Aurintricarboxylic Acid
3	1% CTAB, 1 mM Aurintricarboxylic Acid
4	1% CTAB, 0.5 mM Aurintricarboxylic Acid
5	1% CTAB, 0.2 mM Aurintricarboxylic Acid
6	1% CTAB, 0.1 mM Aurintricarboxylic Acid
7	1% CTAB
8	1% CTACI, 5 mM Aurintricarboxylic Acid
9	1% CTACI, 2 mM Aurintricarboxylic Acid
10	1% CTACI, 1 mM Aurintricarboxylic Acid
11	1% CTACI, 0.5 mM Aurintricarboxylic Acid
12	1% CTACI, 0.2 mM Aurintricarboxylic Acid
13	1% CTACI, 0.1 mM Aurintricarboxylic Acid
14	1% CTACI
15	1% SDS, 5 mM Aurintricarboxylic Acid
16	1% SDS, 2 mM Aurintricarboxylic Acid
17	1% SDS, 1 mM Aurintricarboxylic Acid
18	1% SDS, 0.5 mM Aurintricarboxylic Acid
19	1% SDS, 0.2 mM Aurintricarboxylic Acid
20	1% SDS, 0.1 mM Aurintricarboxylic Acid
21	1% SDS

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L. L. P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

The tubes were incubated at 55°C with mixing (Eppendorf Thermomixer, Model 5436) for 30 minutes. The resulting surfactant-protease complex was solubilized using methods the same as or similar to those described in Example 7. Undigested tissue fragments were removed by centrifugation using methods the same as or similar to those described in Example 7. The supernatant was phenol extracted, isopropanol precipitated and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspended and the integrity of the released nucleic acid were evaluated using methods the same as or similar to those described in Example 6.

As shown in Figure 11, the presence of aurintricarboxylic acid in the reaction composition enhanced the yield of high molecular weight nucleic acid. Reaction compositions containing aurintricarboxylic acid and either CTAB or CTACI released a large amount of high integrity nucleic acid. When SDS was the surfactant in the reaction composition, less nucleic acid was released.

#### **Example 11:**

To examine the ability of other cationic reagents to release high integrity nucleic acid, additional compounds were tested as follows. Slices of liver tissue (80 - 140 mg/sample) were placed into fourteen Eppendorf tubes. Five hundred microliters of a reaction composition comprising 100 mM MES, pH 6, 20 mM CaCl<sub>2</sub>, 0.5 mM aurintricarboxylic acid (e.g., Sigma #A1895), and 1 mg Proteinase K, was added to each tube. One of the cationic reagents shown in Table 11, was added to each tube to a final concentration of 1%. As a control, one tube contained no additional reagent. The tubes were incubated at 60°C for 30 minutes with mixing (Eppendorf Thermomixer,

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

Model 5436). Following the incubation, 200  $\mu$ L of a solution containing 1.75 M NaCl, 29% Tween 20, and 585  $\mu$ g/mL glycogen (resulting in a final concentration of 447 mM NaCl, 7.5% Tween 20, and 149  $\mu$ g/mL glycogen) was added to each tube to dissolve the surfactant:nucleic acid complex. The remaining tissue fragments were removed by centrifugation using methods the same as or similar to those described in Example 7. The supernatant was phenol extracted, isopropanol precipitated, and ethanol washed using methods the same as or similar to those described in Example 6. The washed nucleic acid pellets were resuspended in 100  $\mu$ L 10 mM Tris, pH 8.0, 0.1 mM EDTA, and 1 unit/ $\mu$ L Rnasin. The amount of nucleic acid released and its integrity were evaluated using methods the same as or similar to those described in Example 6.

Table 11.

Tube	Cationic Reagent
1	dodecyltrimethylammonium bromide (DTAB)
2	tetradecyltrimethylammonium bromide (TTAB)
3	CTAB
4	CTACI
5, 6	HTAB
7	Mackernium 006 (polyquaternium 6)
8	Mackernium (Olealkonium chloride)
9	Mackernium NLE (Quaternium-84)
10	Mackernium 007 (Polyquaternium 7)
11	Mackernium Stearalkonium SDC85 Chloride
12	Benzalkonium chloride
13	SDS

As shown in Figure 12, all of the cationic reagents tested, except for Mackernium 006 and 007, released high integrity nucleic acid. Of the eleven cationic reagents tested, nine were surfactants and two, Mackernium 006 and 007, were cationic polymers. This result demonstrates that, under these conditions, all of the cationic

LAW OFFICES

FINNEGAN, HENDERSON,  
 FARABOW, GARRETT  
 & DUNNER, L.L.P.  
 STANFORD RESEARCH PARK  
 700 HANSEN WAY  
 PALO ALTO, CALIF. 94304  
 650-849-6600

surfactants when used in conjunction with Proteinase K, effectively released nucleic acid from the sample.

5 While the present invention has been described in terms of these exemplary embodiments, the skilled artisan will readily understand that numerous variations and modifications of these exemplary embodiments are possible without undue experimentation. All such variations and modifications are within the intended scope of the claimed invention.

10  
20  
30  
40  
50  
60  
70  
80  
90  
100  
110  
120  
130  
140  
150  
160  
170  
180  
190  
200  
210  
220  
230  
240  
250  
260  
270  
280  
290  
300  
310  
320  
330  
340  
350  
360  
370  
380  
390  
400  
410  
420  
430  
440  
450  
460  
470  
480  
490  
500  
510  
520  
530  
540  
550  
560  
570  
580  
590  
600  
610  
620  
630  
640  
650  
660  
670  
680  
690  
700  
710  
720  
730  
740  
750  
760  
770  
780  
790  
800  
810  
820  
830  
840  
850  
860  
870  
880  
890  
900  
910  
920  
930  
940  
950  
960  
970  
980  
990  
1000

LAW OFFICES

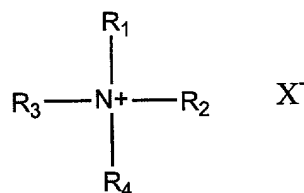
FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L. L. P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

What is claimed is:

1. A composition for releasing nucleic acids from a biological sample comprising: at least one cationic surfactant, at least one protease, and a buffer.

5 2. The composition of claim 1, wherein the at least one cationic surfactant is protonated under the conditions used.

3. The composition of claim 1, wherein the at least one cationic surfactant has the structure:



10 wherein  $R_1$ ,  $R_2$ ,  $R_3$ , and  $R_4$  are independently selected from the group consisting of:  $-H$ ; an alkyl group comprising between one and twenty carbon atoms; and an aryl group comprising between six to twenty-six carbon atoms.

15 4. The composition of claim 3, wherein the cationic surfactant is an alkyltrimethyl ammonium salt, where  $R_1$ ,  $R_2$ , and  $R_3$  are methyl groups, and  $R_4$  is an alkyl group comprising 6, 8, 10, 12, 14, 16, or 18 carbon atoms.

5. The composition of claim 4, where the cation of the alkyltrimethyl ammonium salt is selected from the group consisting of cetyltrimethylammonium, hexadecyltrimethylammonium, tetradecyltrimethylammonium, dodecyltrimethylammonium, and lauryl trimethylammonium.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L. L. P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600



6. The composition of claim 4, where the anion (X<sup>-</sup>) of the alkyltrimethyl ammonium salt is selected from the group including bromide, chloride, iodide, hydroxide, nitrate, sulfate, phosphate, formate, acetate, propionate, oxalate, malonate, succinate, or citrate.

5 7. The composition of claim 3, wherein the at least one cationic surfactant is a benzyldimethyl-*n*-alkylammonium salt, where R<sub>1</sub> and R<sub>2</sub> are methyl groups, R<sub>3</sub> is an aryl group comprising six carbon atoms, and R<sub>4</sub> is an alkyl group comprising 6, 8, 10, 12, 14, 16, or 18 carbon atoms.

10 8. The composition of claim 7, where the anion of the benzyldimethyl-*n*-alkylammonium salt is selected from the group consisting of bromide, chloride, iodide, hydroxide, nitrate, sulfate, phosphate, formate, acetate, propionate, oxalate, malonate, succinate, and citrate.

9. The composition of claim 1, wherein the at least one protease is selected from the group consisting of subtilisins, subtilases, and alkaline serine proteases.

15 10. The composition of claim 9, wherein the at least one protease is selected from the group consisting of Proteinase K, Proteinase R, Proteinase T, Subtilisin DY, an alkaline serine protease from *Streptomyces griseus* or *Bacillus licheniformis*, Dispase, subtilisin Carlsberg, subtilopeptidase A, and thermolysin.

11. The composition of claim 9, wherein the protease is Proteinase K.

12. The composition of claim 9, wherein the protease is thermolysin or a thermostable protease.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

13. The composition of claim 12, wherein the protease is from *Thermus* Rt41A or *Bacillus thermoproteolyticus rokko*.

14. The composition of claim 1, further comprising calcium chloride.

15. The composition of claim 1, wherein the buffer maintains the pH between pH 7 and pH 9.

16. The composition of claim 1, wherein the buffer maintains the pH between pH 5 and pH 7.

17. The composition of claim 1, further comprising a ribonuclease inhibitor.

18. The composition of claim 17, where in the at least one ribonuclease inhibitor comprises aurintricarboxylic acid, vanadylate ribonucleoside complexes, phenylglyoxal, p-hydroxyphenylglyoxal, polyamines, spermidine, 9-aminoacridine, iodoacetate, bentonite, poly[2'-O-(2,4-dinitrophenyl)]poly(adenylic acid), zinc sulfate, bromopyruvic acid, formamide, copper, or zinc.

19. The composition of claim 18, wherein the at least one ribonuclease inhibitor comprises aurintricarboxylic acid.

20. The composition of claim 1, wherein the cationic surfactant is cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACI), hexadecyltrimethylammonium bromide, or hexadecyltrimethylammonium chloride; the protease is Proteinase K; the buffer maintains the pH between pH 5 and pH 7; and further comprising aurintricarboxylic acid.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

21. The composition of claim 20, further comprising at least one solubilizing agent for enhancing the solubility or permeability of the sample.

22. The composition of claim 21, wherein the solubilizing agent is 1-methyl 2  
5 dimethylsulfoxide.

23. The composition of claim 1, further comprising a deoxyribonuclease inhibitor.

24. The composition of claim 23, wherein the at least one deoxyribonuclease inhibitor comprises a divalent cation chelator.

10 25. The composition of claim 24, wherein the chelator is EDTA, EGTA, of DPTA.

26. A method for isolating nucleic acids from a biological sample comprising:  
  
combining the sample with at least one cationic surfactant, at least one  
protease, and a buffer, to form a reaction composition;  
  
15 incubating the reaction composition at a temperature suitable for releasing nucleic acid from the biological sample; and  
  
isolating the released nucleic acid.

27. The method of claim 26, further comprising adding a second surfactant and a salt.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

28. The method of claim 27, wherein the second surfactant comprises a nonionic surfactant.

29. The method of claim 28, wherein the nonionic surfactant is polyoxyethylene(20)sorbitan monolaurate (Tween 20), Tween 80, Triton X-100, Triton X-114, Triton X-305, Triton X-405, Brij-35, Brij-56, Brij-58, *n*-Decyl- $\beta$ -D-glucopyranoside, *n*-Dodecyl- $\beta$ -D-maltoside, *n*-Hexyl- $\beta$ -D-glucopyranoside, *n*-Octyl- $\beta$ -D-glucopyranoside, *n*-Tetradecyl- $\beta$ -D-maltoside, alkyl glycosides, Glucamides, MEGA-10, MEGA-9, MEGA-8, Genapol X-80, Genapol X-10, Thesit, Lubrol PX, Genapol C-100, Pluronic F-127, APO-10, APO-12, Big CHAP, Digitonin, or polyethyleneglycol-*p*-isooctylphenyl ether (NP-40).

30. The method of claim 27, wherein the second surfactant comprises Tween 20 and the salt comprises a sodium salt.

31. The method of claim 26, wherein the biological sample comprises whole tissue.

32. The method of claim 26, comprising the composition of claim 3.

33. The method of claim 26, comprising the composition of claim 4.

34. The method of claim 26, wherein the at least one cationic surfactant comprises cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACl), hexadecyltrimethylammonium bromide, or hexadecyltrimethylammonium chloride, and the at least one protease comprises Proteinase K.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

35. The method of claim 26, further comprising exposing the released nucleic acid to at least one organic solvent.

36. The method of claim 35, wherein exposing the nucleic acid to at least one organic solvent comprises extracting the nucleic acids, precipitating the nucleic acids, or  
5 both extracting and precipitating the nucleic acids.

37. The method of claim 26, further comprising adding a solid phase component capable of binding the released nucleic acid.

38. The method of claim 26, further comprising adding a chaotropic salt and at least one organic solvent to precipitate the released nucleic acid.

10 39. The method of claim 38, wherein the at least one organic solvent comprises ethanol, isopropanol, or acetone.

40. The method of claim 38, wherein the chaotropic salt comprises guanidinium thiocyanate, guanidine hydrochloride, sodium thiocyanate, sodium perchlorate, or sodium iodide.

15 41. The method of claim 26, further comprising adding a polymer.

42. The method of claim 41, wherein the polymer is a polyethylene glycol.

43. The method of claim 26, further comprising adding a divalent cation capable of precipitating the nucleic acid.

44. The method of claim 43, wherein the divalent cation is zinc.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

45. The method of claim 26, wherein the nucleic acid is ribonucleic acid.

46. The method of claim 45, wherein the reaction composition further comprises a ribonuclease inhibitor.

47. The method of claim 46, wherein the ribonuclease inhibitor comprises  
5 aurintricarboxylic acid, vanadylate ribonucleoside complexes, phenylglyoxal, p-  
hydroxyphenylglyoxal, polyamines, spermidine, 9-aminoacridine, iodoacetate, bentonite,  
poly[2'-O-(2,4-dinitrophenyl)]poly(adenylic acid), zinc sulfate, bromopyruvic acid,  
formamide, copper, or zinc.

48. The method of claim 47, wherein the ribonuclease inhibitor is  
10 aurintricarboxylic acid.

49. The method of claim 45, wherein the reaction composition is incubated at  
a temperature of less than 60° C.

50. The method of claim 49, wherein the reaction composition is incubated at  
a temperature between 40° C and 50° C.

51. The method of claim 45, wherein the reaction composition has a pH of  
15 less than 8.0.

52. The method of claim 51, wherein the reaction composition has a pH  
between 5.0 and 7.0.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

53. The method of claim 52, wherein the reaction composition is incubated at a temperature between 40° C and 50° C, and wherein the reaction composition further comprises aurintricarboxylic acid.

54. A method for releasing nucleic acids from a biological sample comprising:  
 5 combining the sample with at least one cationic surfactant, at least one protease, and a buffer, to form a reaction composition; and  
 incubating the reaction composition at a temperature suitable for releasing the nucleic acids from the biological sample.

55. The method of claim 54, further comprising adding a second surfactant  
 10 and a salt.

56. The method of claim 55, wherein the second surfactant comprises a nonionic surfactant.

57. The method of claim 56, wherein the nonionic surfactant is polyoxyethylene(20)sorbitan monolaurate (Tween 20), Tween 80, Triton X-100, Triton  
 15 X-114, Triton X-305, Triton X-405, Brij-35, Brij-56, Brij-58, *n*-Decyl-β-D-glucopyranoside, *n*-Dodecyl-β-D-maltoside, *n*-Hexyl-β-D-glucopyranoside, *n*-Octyl-β-D-glucopyranoside, *n*-Tetradecyl-β-D-maltoside, alkyl glycosides, Glucamides, MEGA-10, MEGA-9, MEGA-8, Genapol X-80, Genapol X-10, Thesit, Lubrol PX, Genapol C-100, Pluronic F-127, APO-10, APO-12, Big CHAP, Digitonin, or polyethyleneglycol-*p*-isooctylphenyl ether (NP-40).

58. The method of claim 55, wherein the second surfactant comprises Tween 20 and the salt comprises a sodium salt.

59. The method of claim 54, wherein the biological sample comprises whole tissue.

5 60. The method of claim 54, comprising the composition of claim 3.

61. The method of claim 54, comprising the composition of claim 4.

10 62. The method of claim 54, wherein the at least one cationic surfactant comprises cetyltrimethylammonium bromide (CTAB), cetyltrimethylammonium chloride (CTACI), hexadecyltrimethylammonium bromide, or hexadecyltrimethylammonium chloride, and the at least one protease comprises Proteinase K.

63. The method of claim 54, wherein the nucleic acid is ribonucleic acid.

64. The method of claim 63, wherein the reaction composition further comprises a ribonuclease inhibitor.

15 65. The method of claim 64, wherein the ribonuclease inhibitor comprises aurintricarboxylic acid, vanadylate ribonucleoside complexes, phenylglyoxal, p-hydroxyphenylglyoxal, polyamines, spermidine, 9-aminoacridine, iodoacetate, bentonite, poly[2'-O-(2,4-dinitrophenyl)]poly(adenylic acid), zinc sulfate, bromopyruvic acid, formamide, copper, or zinc.

66. The method of claim 65, wherein the ribonuclease inhibitor is aurintricarboxylic acid.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600



67. The method of claim 63, wherein the reaction composition is incubated at a temperature of less than 60° C.

68. The method of claim 67, wherein the reaction composition is incubated at a temperature between 40° C and 50° C.

5 69. The method of claim 63, wherein the reaction composition has a pH of less than 8.0.

70. The method of claim 69, wherein the reaction composition has a pH between 5.0 and 7.0.

10 71. The method of claim 70, wherein the reaction composition is incubated at a temperature between 40° C and 50° C, and wherein the reaction composition further comprises aurintricarboxylic acid.

72. A kit for obtaining nucleic acid from a biological sample comprising at least one cationic surfactant and at least one protease.

73. The kit of claim 72, comprising the at least one surfactant of claim 3.

15 74. The kit of claim 73, wherein the at least one cationic surfactant comprises cetyltrimethylammonium bromide, cetyltrimethylammonium chloride, hexadecyltrimethylammonium bromide, or hexadecyltrimethylammonium chloride.

75. The kit of claim 72, wherein the at least one protease is selected from the group consisting of subtilisins, subtilases and alkaline serine proteases.

76. The kit of claim 75, wherein the at least one protease is Proteinase K.

LAW OFFICES

FINNÉGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.

STANFORD RESEARCH PARK

700 HANSEN WAY

PALO ALTO, CALIF. 94304

650-849-6600

20

77. The kit of claim 72, further comprising a second surfactant and a salt.

78. The kit of claim 72, further comprising at least one organic solvent for extracting the nucleic acids, precipitating the nucleic acids, or both extracting and precipitating the nucleic acids.

5 79. The kit of claim 78, wherein the organic solvent for extracting nucleic acids comprises phenol and the organic solvent for precipitating nucleic acids comprises isopropanol or ethanol.

80. The kit of claim 72, further comprising a solid phase component.

10 81. The kit of claim 72, further comprising a chaotropic salt and an organic solvent.

82. The kit of claim 72, further comprising a polymer.

83. The kit of claim 72, further comprising a divalent cation capable of precipitating nucleic acid.

84. The kit of claim 72, further comprising at least one ribonuclease inhibitor.

15 85. The kit of claim 84, wherein the at least one ribonuclease inhibitor is aurintricarboxylic acid, vanadylate ribonucleoside complexes, phenylglyoxal, p-hydroxyphenylglyoxal, polyamines, spermidine, 9-aminoacridine, iodoacetate, Bentonite, poly[2'-O-(2,4-dinitrophenyl)]poly(adenylic acid), zinc sulfate, bromopyruvic acid, formamide, copper, or zinc.

86. The kit of claim 72, further comprising at least one solubilizing agent.

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L.L.P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

20

87. The kit of claim 86, wherein the solubilizing agent comprises 1-methyl 2 pyrolidinone, N-methyl pyrolidinone, pyrolidinone, dimethylformamide, or dimethylsulfoxide.

5

Variable	Unit	Value	Variable	Unit	Value
Age	Years	25.5	Age	Years	25.5
Age <sup>2</sup>	Years <sup>2</sup>	650.25	Age <sup>2</sup>	Years <sup>2</sup>	650.25
Age <sup>3</sup>	Years <sup>3</sup>	16937.5	Age <sup>3</sup>	Years <sup>3</sup>	16937.5
Age <sup>4</sup>	Years <sup>4</sup>	432812.5	Age <sup>4</sup>	Years <sup>4</sup>	432812.5
Age <sup>5</sup>	Years <sup>5</sup>	11145625	Age <sup>5</sup>	Years <sup>5</sup>	11145625
Age <sup>6</sup>	Years <sup>6</sup>	284140625	Age <sup>6</sup>	Years <sup>6</sup>	284140625
Age <sup>7</sup>	Years <sup>7</sup>	7253593750	Age <sup>7</sup>	Years <sup>7</sup>	7253593750
Age <sup>8</sup>	Years <sup>8</sup>	185339843750	Age <sup>8</sup>	Years <sup>8</sup>	185339843750
Age <sup>9</sup>	Years <sup>9</sup>	4728496093750	Age <sup>9</sup>	Years <sup>9</sup>	4728496093750
Age <sup>10</sup>	Years <sup>10</sup>	120212406250000	Age <sup>10</sup>	Years <sup>10</sup>	120212406250000
Age <sup>11</sup>	Years <sup>11</sup>	3065310468750000	Age <sup>11</sup>	Years <sup>11</sup>	3065310468750000
Age <sup>12</sup>	Years <sup>12</sup>	77632761718750000	Age <sup>12</sup>	Years <sup>12</sup>	77632761718750000
Age <sup>13</sup>	Years <sup>13</sup>	1965841503125000000	Age <sup>13</sup>	Years <sup>13</sup>	1965841503125000000
Age <sup>14</sup>	Years <sup>14</sup>	50148439078125000000	Age <sup>14</sup>	Years <sup>14</sup>	50148439078125000000
Age <sup>15</sup>	Years <sup>15</sup>	1278710976953125000000	Age <sup>15</sup>	Years <sup>15</sup>	1278710976953125000000
Age <sup>16</sup>	Years <sup>16</sup>	32467774423828125000000	Age <sup>16</sup>	Years <sup>16</sup>	32467774423828125000000
Age <sup>17</sup>	Years <sup>17</sup>	824142115019531250000000	Age <sup>17</sup>	Years <sup>17</sup>	824142115019531250000000
Age <sup>18</sup>	Years <sup>18</sup>	20907294990507812500000000	Age <sup>18</sup>	Years <sup>18</sup>	20907294990507812500000000
Age <sup>19</sup>	Years <sup>19</sup>	532684469762656250000000000	Age <sup>19</sup>	Years <sup>19</sup>	532684469762656250000000000
Age <sup>20</sup>	Years <sup>20</sup>	13567111744066406250000000000	Age <sup>20</sup>	Years <sup>20</sup>	13567111744066406250000000000
Age <sup>21</sup>	Years <sup>21</sup>	344209804745695312500000000000	Age <sup>21</sup>	Years <sup>21</sup>	344209804745695312500000000000
Age <sup>22</sup>	Years <sup>22</sup>	8749445519272656250000000000000	Age <sup>22</sup>	Years <sup>22</sup>	8749445519272656250000000000000
Age <sup>23</sup>	Years <sup>23</sup>	22178558349604687500000000000000	Age <sup>23</sup>	Years <sup>23</sup>	22178558349604687500000000000000
Age <sup>24</sup>	Years <sup>24</sup>	564442456990117187500000000000000	Age <sup>24</sup>	Years <sup>24</sup>	564442456990117187500000000000000
Age <sup>25</sup>	Years <sup>25</sup>	14111061424752929687500000000000000	Age <sup>25</sup>	Years <sup>25</sup>	14111061424752929687500000000000000
Age <sup>26</sup>	Years <sup>26</sup>	356786586023426562500000000000000000	Age <sup>26</sup>	Years <sup>26</sup>	356786586023426562500000000000000000
Age <sup>27</sup>	Years <sup>27</sup>	9020451186598300000000000000000000000	Age <sup>27</sup>	Years <sup>27</sup>	9020451186598300000000000000000000000
Age <sup>28</sup>	Years <sup>28</sup>	228531630741552000000000000000000000000	Age <sup>28</sup>	Years <sup>28</sup>	228531630741552000000000000000000000000
Age <sup>29</sup>	Years <sup>29</sup>	5818281378279808000000000000000000000000	Age <sup>29</sup>	Years <sup>29</sup>	5818281378279808000000000000000000000000
Age <sup>30</sup>	Years <sup>30</sup>	145457034457000000000000000000000000000000	Age <sup>30</sup>	Years <sup>30</sup>	145457034457000000000000000000000000000000
Age <sup>31</sup>	Years <sup>31</sup>	3636425861425000000000000000000000000000000	Age <sup>31</sup>	Years <sup>31</sup>	3636425861425000000000000000000000000000000
Age <sup>32</sup>	Years <sup>32</sup>	91516857295800000000000000000000000000000000	Age <sup>32</sup>	Years <sup>32</sup>	91516857295800000000000000000000000000000000
Age <sup>33</sup>	Years <sup>33</sup>	231842718359040000000000000000000000000000000	Age <sup>33</sup>	Years <sup>33</sup>	231842718359040000000000000000000000000000000
Age <sup>34&lt;/</sup>					

LAW OFFICES

FINNEGAN, HENDERSON,  
FARABOW, GARRETT  
& DUNNER, L. L. P.  
STANFORD RESEARCH PARK  
700 HANSEN WAY  
PALO ALTO, CALIF. 94304  
650-849-6600

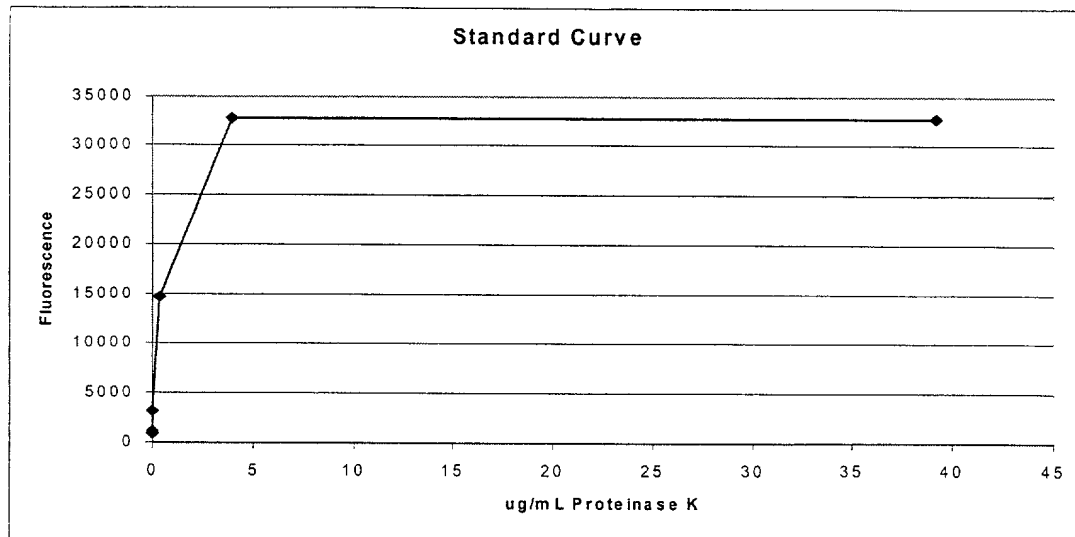
ABSTRACT

The invention provides compositions and methods for releasing and for isolating nucleic acids from biological samples, preferably from whole tissue, using cationic surfactants and proteases. The surfactant-protease combinations, when used with whole tissue, macerate the tissue, lyse individual cells, release nucleic acids, and inactivate nucleases. Kits for isolating nucleic acids from biological samples, particularly from whole tissue, are also provided.

5

10

11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
66  
67  
68  
69  
70  
71  
72  
73  
74  
75  
76  
77  
78  
79  
80  
81  
82  
83  
84  
85  
86  
87  
88  
89  
90  
91  
92  
93  
94  
95  
96  
97  
98  
99  
100  
101  
102  
103  
104  
105  
106  
107  
108  
109  
110  
111  
112  
113  
114  
115  
116  
117  
118  
119  
120  
121  
122  
123  
124  
125  
126  
127  
128  
129  
130  
131  
132  
133  
134  
135  
136  
137  
138  
139  
140  
141  
142  
143  
144  
145  
146  
147  
148  
149  
150  
151  
152  
153  
154  
155  
156  
157  
158  
159  
160  
161  
162  
163  
164  
165  
166  
167  
168  
169  
170  
171  
172  
173  
174  
175  
176  
177  
178  
179  
180  
181  
182  
183  
184  
185  
186  
187  
188  
189  
190  
191  
192  
193  
194  
195  
196  
197  
198  
199  
200  
201  
202  
203  
204  
205  
206  
207  
208  
209  
210  
211  
212  
213  
214  
215  
216  
217  
218  
219  
220  
221  
222  
223  
224  
225  
226  
227  
228  
229  
230  
231  
232  
233  
234  
235  
236  
237  
238  
239  
240  
241  
242  
243  
244  
245  
246  
247  
248  
249  
250  
251  
252  
253  
254  
255  
256  
257  
258  
259  
260  
261  
262  
263  
264  
265  
266  
267  
268  
269  
270  
271  
272  
273  
274  
275  
276  
277  
278  
279  
280  
281  
282  
283  
284  
285  
286  
287  
288  
289  
290  
291  
292  
293  
294  
295  
296  
297  
298  
299  
300  
301  
302  
303  
304  
305  
306  
307  
308  
309  
310  
311  
312  
313  
314  
315  
316  
317  
318  
319  
320  
321  
322  
323  
324  
325  
326  
327  
328  
329  
330  
331  
332  
333  
334  
335  
336  
337  
338  
339  
340  
341  
342  
343  
344  
345  
346  
347  
348  
349  
350  
351  
352  
353  
354  
355  
356  
357  
358  
359  
360  
361  
362  
363  
364  
365  
366  
367  
368  
369  
370  
371  
372  
373  
374  
375  
376  
377  
378  
379  
380  
381  
382  
383  
384  
385  
386  
387  
388  
389  
390  
391  
392  
393  
394  
395  
396  
397  
398  
399  
400  
401  
402  
403  
404  
405  
406  
407  
408  
409  
410  
411  
412  
413  
414  
415  
416  
417  
418  
419  
420  
421  
422  
423  
424  
425  
426  
427  
428  
429  
430  
431  
432  
433  
434  
435  
436  
437  
438  
439  
440  
441  
442  
443  
444  
445  
446  
447  
448  
449  
450  
451  
452  
453  
454  
455  
456  
457  
458  
459  
460  
461  
462  
463  
464  
465  
466  
467  
468  
469  
470  
471  
472  
473  
474  
475  
476  
477  
478  
479  
480  
481  
482  
483  
484  
485  
486  
487  
488  
489  
490  
491  
492  
493  
494  
495  
496  
497  
498  
499  
500  
501  
502  
503  
504  
505  
506  
507  
508  
509  
510  
511  
512  
513  
514  
515  
516  
517  
518  
519  
520  
521  
522  
523  
524  
525  
526  
527  
528  
529  
530  
531  
532  
533  
534  
535  
536  
537  
538  
539  
540  
541  
542  
543  
544  
545  
546  
547  
548  
549  
550  
551  
552  
553  
554  
555  
556  
557  
558  
559  
560  
561  
562  
563  
564  
565  
566  
567  
568  
569  
570  
571  
572  
573  
574  
575  
576  
577  
578  
579  
580  
581  
582  
583  
584  
585  
586  
587  
588  
589  
590  
591  
592  
593  
594  
595  
596  
597  
598  
599  
600  
601  
602  
603  
604  
605  
606  
607  
608  
609  
610  
611  
612  
613  
614  
615  
616  
617  
618  
619  
620  
621  
622  
623  
624  
625  
626  
627  
628  
629  
630  
631  
632  
633  
634  
635  
636  
637  
638  
639  
640  
641  
642  
643  
644  
645  
646  
647  
648  
649  
650  
651  
652  
653  
654  
655  
656  
657  
658  
659  
660  
661  
662  
663  
664  
665  
666  
667  
668  
669  
670  
671  
672  
673  
674  
675  
676  
677  
678  
679  
680  
681  
682  
683  
684  
685  
686  
687  
688  
689  
690  
691  
692  
693  
694  
695  
696  
697  
698  
699  
700  
701  
702  
703  
704  
705  
706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718  
719  
720  
721  
722  
723  
724  
725  
726  
727  
728  
729  
730  
731  
732  
733  
734  
735  
736  
737  
738  
739  
740  
741  
742  
743  
744  
745  
746  
747  
748  
749  
750  
751  
752  
753  
754  
755  
756  
757  
758  
759  
760  
761  
762  
763  
764  
765  
766  
767  
768  
769  
770  
771  
772  
773  
774  
775  
776  
777  
778  
779  
780  
781  
782  
783  
784  
785  
786  
787  
788  
789  
790  
791  
792  
793  
794  
795  
796  
797  
798  
799  
800  
801  
802  
803  
804  
805  
806  
807  
808  
809  
810  
811  
812  
813  
814  
815  
816  
817  
818  
819  
820  
821  
822  
823  
824  
825  
826  
827  
828  
829  
830  
831  
832  
833  
834  
835  
836  
837  
838  
839  
840  
841  
842  
843  
844  
845  
846  
847  
848  
849  
850  
851  
852  
853  
854  
855  
856  
857  
858  
859  
860  
861  
862  
863  
864  
865  
866  
867  
868  
869  
870  
871  
872  
873  
874  
875  
876  
877  
878  
879  
880  
881  
882  
883  
884  
885  
886  
887  
888  
889  
890  
891  
892  
893  
894  
895  
896  
897  
898  
899  
900  
901  
902  
903  
904  
905  
906  
907  
908  
909  
910  
911  
912  
913  
914  
915  
916  
917  
918  
919  
920  
921  
922  
923  
924  
925  
926  
927  
928  
929  
930  
931  
932  
933  
934  
935  
936  
937  
938  
939  
940  
941  
942  
943  
944  
945  
946  
947  
948  
949  
950  
951  
952  
953  
954  
955  
956  
957  
958  
959  
960  
961  
962  
963  
964  
965  
966  
967  
968  
969  
970  
971  
972  
973  
974  
975  
976  
977  
978  
979  
980  
981  
982  
983  
984  
985  
986  
987  
988  
989  
990  
991  
992  
993  
994  
995  
996  
997  
998  
999  
1000  
1001  
1002  
1003  
1004  
1005  
1006  
1007  
1008  
1009  
1010  
1011  
1012  
1013  
1014  
1015  
1016  
1017  
1018  
1019  
1020  
1021  
1022  
1023  
1024  
1025  
1026  
1027  
1028  
1029  
1030  
1031  
1032  
1033  
1034  
1035  
1036  
1037  
1038  
1039  
1040  
1041  
1042  
1043  
1044  
1045  
1046  
1047  
1048  
1049  
1050  
1051  
1052  
1053  
1054  
1055  
1056  
1057  
1058  
1059  
1060  
1061  
1062  
1063  
1064  
1065  
1066  
1067  
1068  
1069  
1070  
1071  
1072  
1073  
1074  
1075  
1076  
1077  
1078  
1079  
1080  
1081  
1082  
1083  
1084  
1085  
1086  
1087  
1088  
1089  
1090  
1091  
1092  
1093  
1094  
1095  
1096  
1097  
1098  
1099  
1100  
1101  
1102  
1103  
1104  
1105  
1106  
1107  
1108  
1109  
1110  
1111  
1112  
1113  
1114  
1115  
1116  
1117  
1118  
1119  
1120  
1121  
1122  
1123  
1124  
1125  
1126  
1127  
1128  
1129  
1130  
1131  
1132  
1133  
1134  
1135  
1136  
1137  
1138  
1139  
1140  
1141  
1142  
1143  
1144  
1145  
1146  
1147  
1148  
1149  
1150  
1151  
1152  
1153  
1154  
1155  
1156  
1157  
1158  
1159  
1160  
1161  
1162  
1163  
1164  
1165  
1166  
1167  
1168  
1169  
1170  
1171  
1172  
1173  
1174  
1175  
1176  
1177  
1178  
1179  
1180  
1181  
1182  
1183  
1184  
1185  
1186  
1187  
1188  
1189  
1190  
1191  
1192  
1193  
1194  
1195  
1196  
1197  
1198  
1199  
1200  
1201  
1202  
1203  
1204  
1205  
1206  
1207  
1208  
1209  
1210  
1211  
1212  
1213  
1214  
1215  
1216  
1217  
1218  
1219  
1220  
1221  
1222  
1223  
1224  
1225  
1226  
1227  
1228  
1229  
1230  
1231  
1232  
1233  
1234  
1235  
1236  
1237  
1238  
1239  
1240  
1241  
1242  
1243  
1244  
1245  
1246  
1247  
1248  
1249  
1250  
1251  
1252  
1253  
1254  
1255  
1256  
1257  
1258  
1259  
1260  
1261  
1262  
1263  
1264  
1265  
1266  
1267  
1268  
1269  
1270  
1271  
1272  
1273  
1274  
1275  
1276  
1277  
1278  
1279  
1280  
1281  
1282  
1283  
1284  
1285  
1286  
1287  
1288  
1289  
1290  
1291  
1292  
1293  
1294  
1295  
1296  
1297  
1298  
1299  
1300  
1301  
1302  
1303  
1304  
1305  
1306  
1307  
1308  
1309  
1310  
1311  
1312  
1313  
1314  
1315  
1316  
1317  
1318  
1319  
1320  
1321  
1322  
1323  
1324  
1325  
1326  
1327  
1328  
1329  
1330  
1331  
1332  
1333  
1334  
1335  
1336  
1337  
1338  
1339  
1340  
1341  
1342  
1343  
1344  
1345  
1346  
1347  
1348  
1349  
1350  
1351  
1352  
1353  
1354  
1355  
1356  
1357  
1358  
1359  
1360  
1361  
1362  
1363  
1364  
1365  
1366  
1367  
1368  
1369  
1370  
1371  
1372  
1373  
1374  
1375  
1376  
1377  
1378  
1379  
1380  
1381  
1382  
1383  
1384  
1385  
1386  
1387  
1388  
1389  
1390  
1391  
1392  
1393  
1394  
1395  
1396  
1397  
1398  
1399  
1400  
1401  
1402  
1403  
1404  
1405  
1406  
1407  
1408  
1409  
1410  
1411  
1412  
1413  
1414  
1415  
1416  
1417  
1418  
1419  
1420  
1421  
1422  
1423  
1424  
1425  
1426  
1427  
1428  
1429  
1430  
1431  
1432  
1433  
1434  
1435  
1436  
1437  
1438  
1439  
1440  
1441  
1442  
1443  
1444  
1445  
1446  
1447  
1448  
1449  
1450  
1451  
1452  
1453  
1454  
1455  
1456  
1457  
1458  
1459  
1460  
1461  
1462  
1463  
1464  
1465  
1466  
1467  
1468  
1469  
1470  
1471  
1472  
1473  
1474  
1475  
1476  
1477  
1478  
1479  
1480  
1481  
1482  
1483  
1484  
1485  
1486  
1487  
1488  
1489  
1490  
1491  
1492  
1493  
1494  
1495  
1496  
1497  
1498  
1499  
1500  
1501  
1502  
1503  
1504  
1505  
1506  
1507  
1508  
1509  
1510  
1511  
1512  
1513  
1514  
1515  
1516  
1517  
1518  
1519  
1520  
1521  
1522  
1523  
1524  
1525  
1526  
1527  
1528  
1529  
1530  
1531  
1532  
1533  
1534  
1535  
1536  
1537  
1538  
1539  
1540  
1541  
1542  
1543  
1544  
1545  
1546  
1547  
1548  
1549  
1550  
1551  
1552  
1553  
1554  
1555  
1556  
1557  
1558  
1559  
1560  
1561  
1562  
1563  
1564  
1565  
1566  
1567  
1568  
1569  
1570  
1571  
1572  
1573  
1574  
1575  
1576  
1577  
1578  
1579  
1580  
1581  
1582  
1583  
1584  
1585  
1586  
1587  
1588  
1589  
1590  
1591  
1592  
1593  
1594  
1595  
1596  
1597  
1598  
1599  
1600  
1601  
1602  
1603  
1604  
1605  
1606  
1607  
1608  
1609  
1610  
1611  
1612  
1613  
1614  
1615  
1616  
1617  
1618  
1619  
1620  
1621  
1622  
1623  
1624  
1625  
1626  
1627  
1628  
1629  
1630  
1631  
1632  
1633  
1634  
1635  
1636  
1637  
1638  
1639  
1640  
1641  
1642  
1643  
1644  
1645  
1646  
1647  
1648  
1649  
1650  
1651  
1652  
1653  
1654  
1655  
1656  
1657  
1658  
1659  
1660  
1661  
1662  
1663  
1664  
1665  
1666  
1667  
1668  
1669  
1670  
1671  
1672  
1673  
1674  
1675  
1676  
1677  
1678  
1679  
1680  
1681  
1682  
1683  
1684  
1685  
1686  
1687  
1688  
1689  
1690  
1691  
1692  
1693  
1694  
1695  
1696  
1697  
1698  
1699  
1700  
1701  
1702  
1703  
1704  
1705  
1706  
1707  
1708  
1709  
1710  
1711  
1712  
1713  
1714  
1715  
1716  
1717  
1718  
1719  
1720  
1721  
1722  
1723  
1724  
1725  
1726  
1727  
1728  
1729  
1730  
1731  
1732  
1733  
1734  
1735  
1736  
1737  
1738  
1739  
1740  
1741  
1742  
1743  
1744  
1745  
1746  
1747  
1748  
1749  
1750  
1751  
1752  
1753  
1754  
1755  
1756  
1757  
1758  
1759  
1760  
1761  
1762  
1763  
1764  
1765  
1766  
1767  
1768  
1769  
1770  
1771  
1772  
1773  
1774  
1775  
1776  
1777  
1778  
1779  
1780  
1781  
1782  
1783  
1784  
1785  
1786  
1787  
1788  
1789  
1790  
1791  
1792  
1793  
1794  
1795  
1796  
1797  
1798  
1799  
1800  
1801  
1802  
1803  
1804  
1805  
1806  
1807  
1808  
1809  
1810  
1811  
1812  
1813  
1814  
1815  
1816  
1817  
1818  
1819  
1820  
1821  
1822  
1823  
1824  
1825  
1826  
1827  
1828  
1829  
1830  
1831  
1832  
1833  
1834  
1835  
1836  
1837  
1838  
1839  
1840  
1841  
1842  
1843  
1844  
1845  
1846  
1847  
1848  
1849  
1850  
1851  
1852  
1853  
1854  
1855  
1856  
1857  
1858  
1859  
1860  
1861  
1862  
1863  
1864  
1865  
1866  
1867  
1868  
1869  
1870  
1871  
1872  
1873  
1874  
1875  
1876  
1877  
1878  
1879  
1880  
1881  
1882  
1883  
1884  
1885  
1886  
1887  
1888  
1889  
1890  
1891  
1892  
1893  
1894  
1895  
1896  
1897  
1898  
1899  
1900  
1901  
1902  
1903  
1904  
1905  
1906  
1907  
1908  
1909  
1910  
1911  
1912  
1913  
1914  
1915  
1916  
1917  
1918  
1919  
1920  
1921  
1922  
1923  
1924  
1925  
1926  
1927  
1928  
1929  
1930  
1931  
1932  
1933  
1934  
1935  
1936  
1937  
1938  
1939  
1940  
1941  
1942  
1943  
1944  
1945  
1946  
1947  
1948  
1949  
1950  
1951  
1952  
1953  
1954  
1955  
1956  
1957  
1958  
1959  
1960  
1961  
1962  
1963  
1964  
1965  
1966  
1967  
1968  
1969  
1970  
1971  
1972  
1973  
1974  
1975  
1976  
1977  
1978  
1979  
1980  
1981  
1982  
1983  
1984  
1985  
1986  
1987  
1988  
1989  
1990  
1991  
1992  
1993  
1994  
1995  
1996  
1997  
1998  
1999  
2000  
2001  
2002  
2003  
2004  
2005  
2006  
2007  
2008  
2009  
2010  
2011  
2012  
2013  
2014  
2015  
2016  
2017  
2018  
2019  
2020  
2021  
2022  
2023  
2024  
2025  
2026  
2027  
2028  
2029  
2030  
2031  
2032  
2033  
2034  
2035  
2036  
2037  
2038  
2039  
2040  
2041  
2042  
2043  
2044  
2045  
2046  
2047  
2048  
2049  
2050  
2051  
2052  
2053  
2054  
2055  
2056  
2057  
2058  
2059  
2060  
2061  
2062  
2063  
2064  
2065  
2066  
2067  
2068  
2069  
2070  
2071  
2072  
2073  
2074  
2075  
2076  
2077  
2078  
2079  
2080  
2081  
2082  
2083  
2084  
2085  
2086  
2087  
2088  
2089  
2090  
2091  
2092  
2093  
2094  
2095  
2096  
2097  
2098  
2099  
2100  
2101  
2102  
2103  
2104  
2105  
2106  
2107  
2108  
2109  
2110  
2111  
2112  
2113  
2114  
2115  
2116  
2117  
2118  
2119  
2120  
2121  
2122  
2123  
2124  
2125  
2126  
2127  
2128  
2129  
2130  
2131  
2132  
2133  
2134  
2135  
2136  
2137  
2138  
2139  
2140  
2141  
2142  
2143  
2144  
2145  
2146  
2147  
2148  
2149  
2150  
2151  
2152  
2153  
2154  
2155  
2156  
2157  
2158  
2159  
2160  
2161  
2162  
2163  
2164  
2165  
2166  
2167  
2168  
2169  
2170  
2171  
2172  
2173  
2174  
2175  
2176  
2177  
2178  
2179  
2180  
2181  
2182  
2183  
2184  
2185  
2186  
2187  
2188  
2189  
2190  
2191  
2192  
2193  
2194  
2195  
2196  
2197  
2198  
2199  
2200  
2201  
2202  
2203



**Figure 1**

### 20 ug/mL Proteinase K

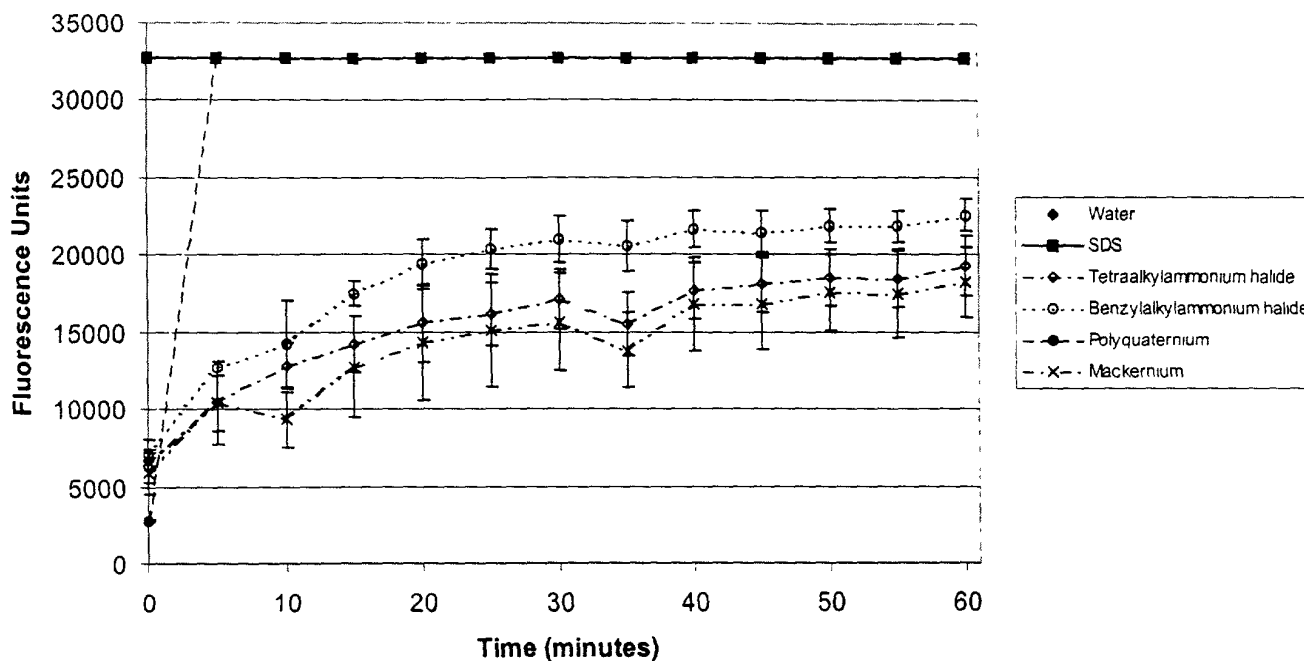


Figure 2A

### 2.5 ug/mL Proteinase K

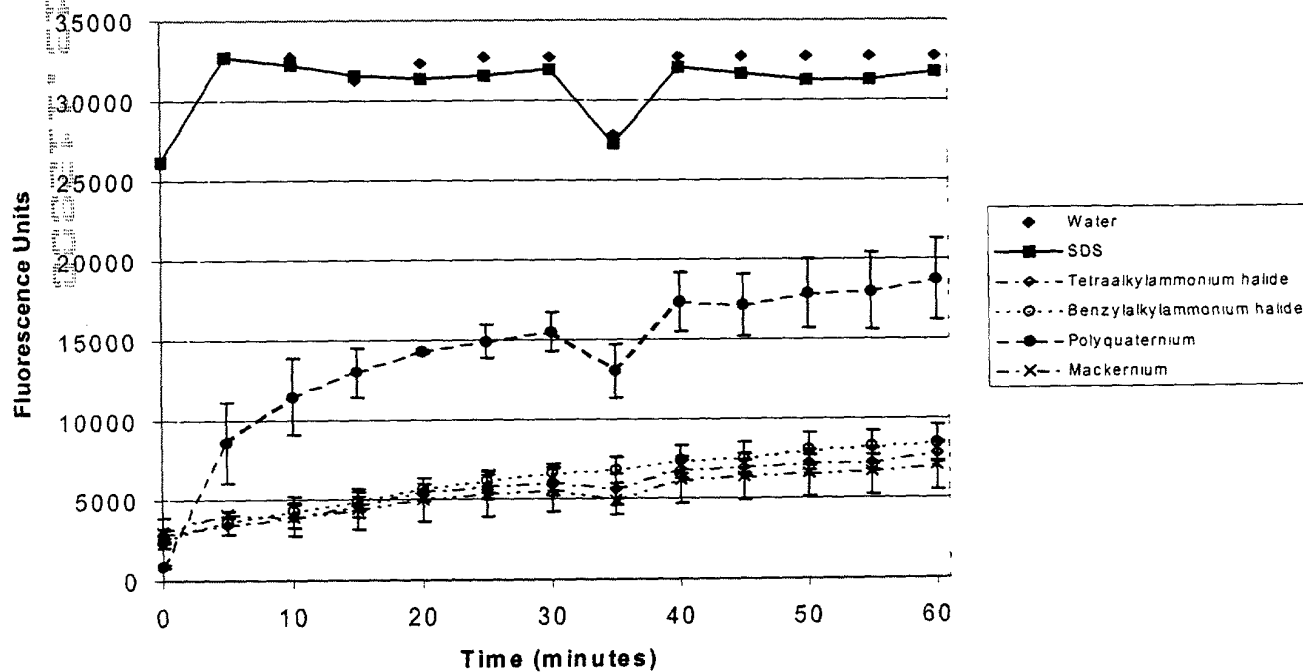


Figure 2B

# 1.25 ug/mL Proteinase K

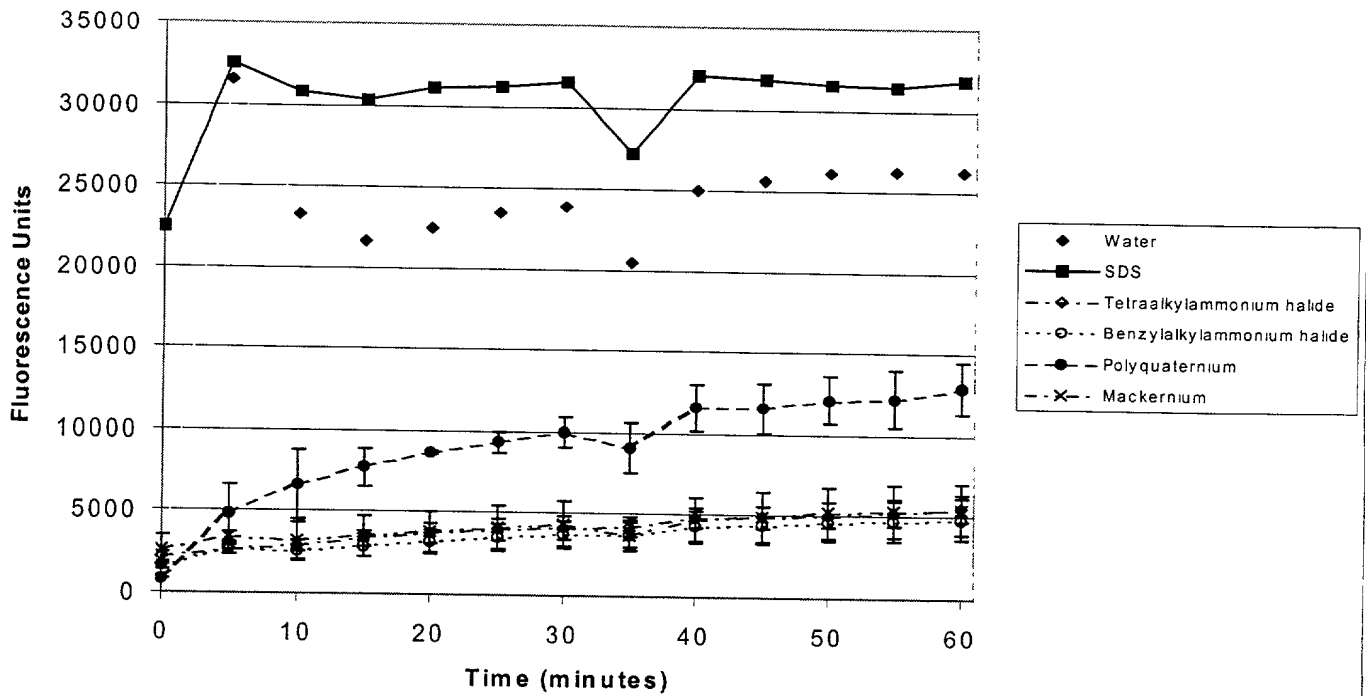
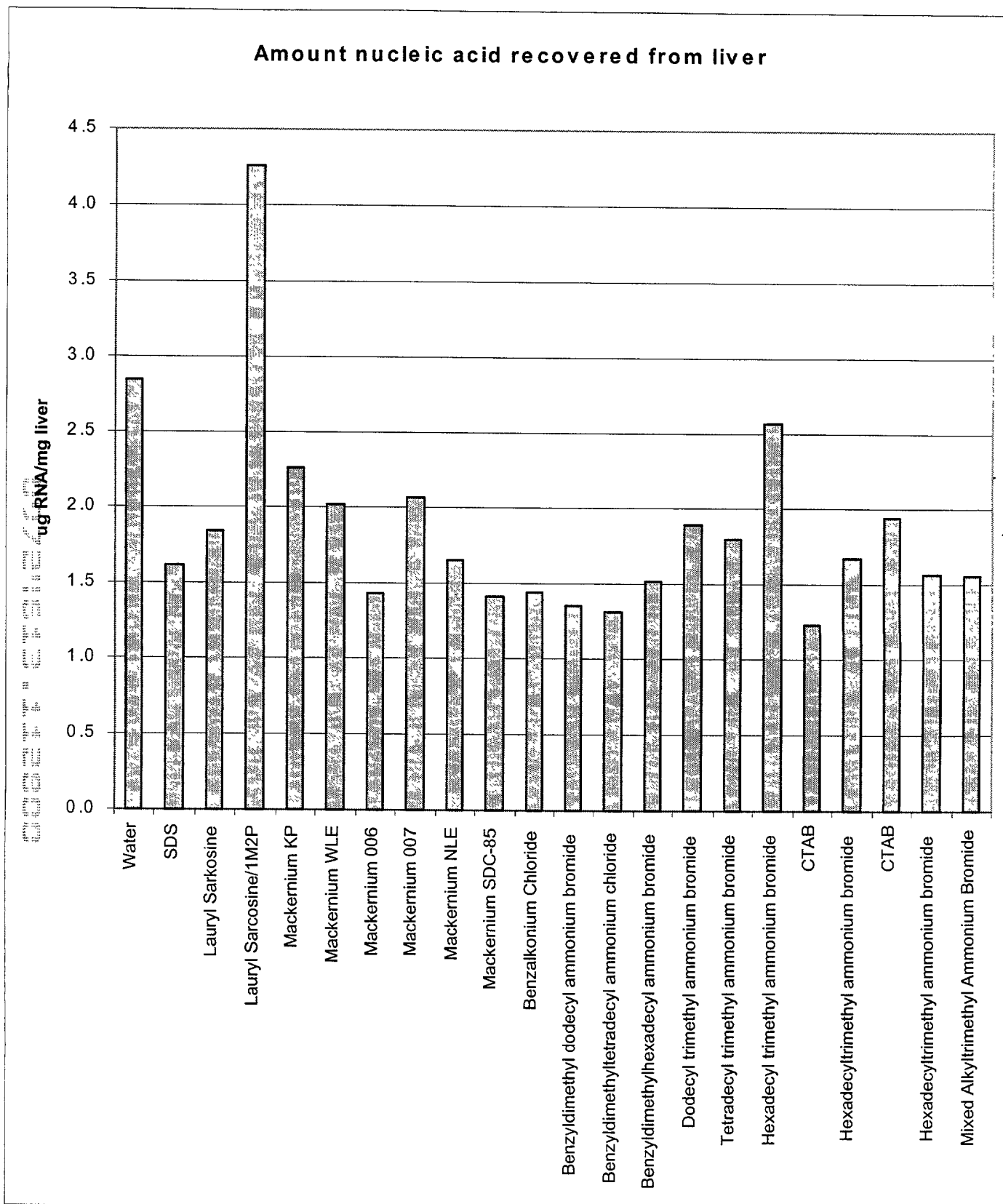


Figure 2C



**Figure 3**



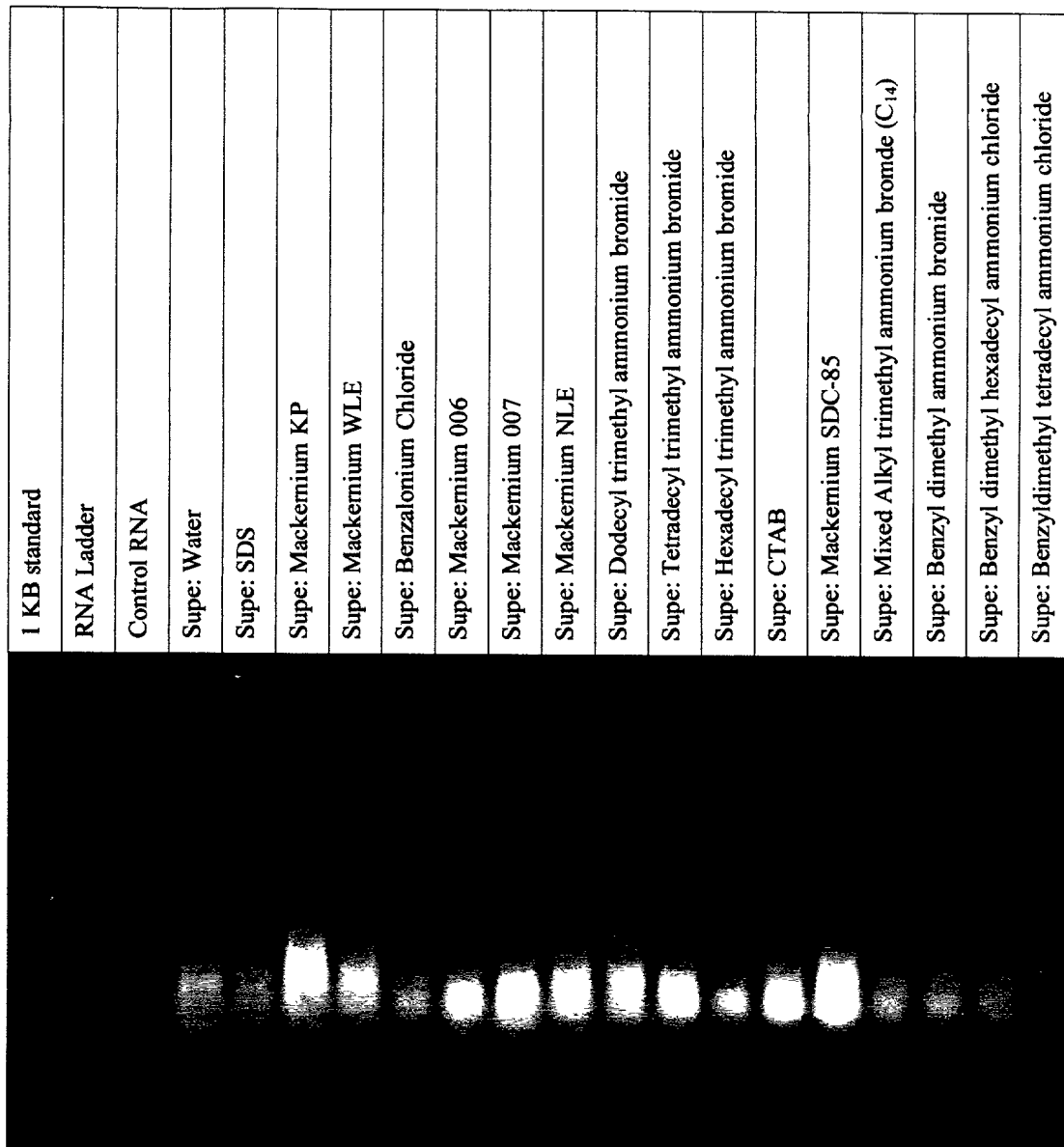
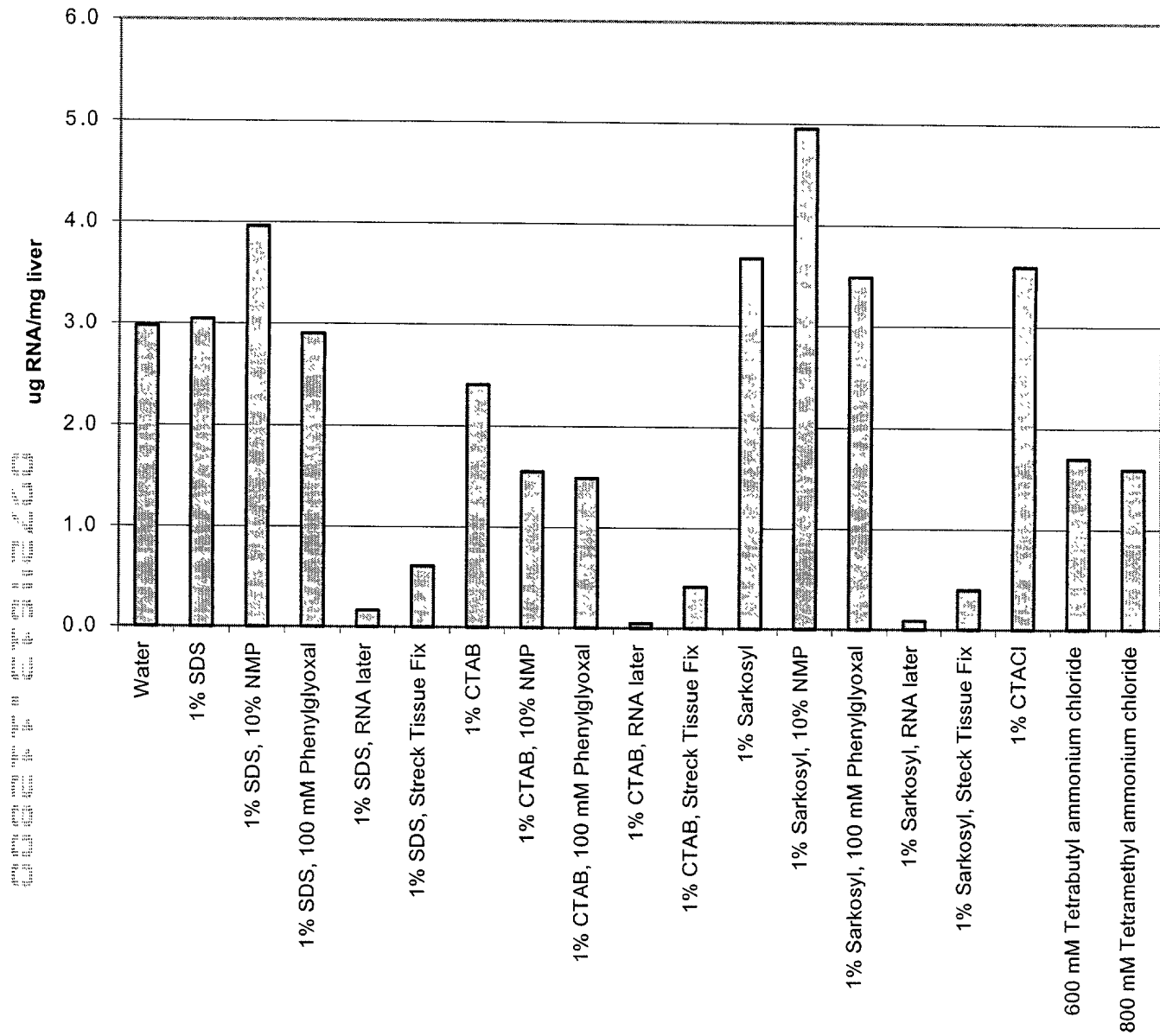


Figure 4

Supe: Hexadecyl trimethyl ammonium bromide	
Supe: CTAB	
Supe: Hexadecyl trimethyl ammonium bromide	
Supe: Lauryl sarcosine	
Supe: Lauryl sarcosine/1-methyl 2-pyrrolidone	
Pellet: Water	
Pellet: SDS	
Pellet: Mackernium KP	
Pellet: Mackernium WLE	
Pellet: Benzalonium Chloride	
Pellet: Mackernium 006	
Pellet: Mackernium 007	
Pellet: Mackernium NLE	
Pellet: Dodecyl trimethyl ammonium bromide	
Pellet: Tetradecyl trimethyl ammonium bromide	
Pellet: Hexadecyl trimethyl ammonium bromide	
Pellet: CTAB	
1 KB standard	
RNA Ladder	
Control RNA	

Figure 4 (cont.)

**Amount nucleic acid released from liver  
2 mg/mL Proteinase K 45°C 20 minutes plus**



**Figure 5**

1KB DNA Standard	
RNA Ladder	
Human RNA control	
	No detergent
	1% SDS
10% 1 Methyl 2-pyrrolidinone	1% SDS
100 mM phenylglyoxal	1% SDS
RNA Later	1% SDS
Streck Tissue Fixative	1% SDS
	1% CTAB
10% 1 Methyl 2-pyrrolidinone	1% CTAB
100 mM phenylglyoxal	1% CTAB
RNA Later	1% CTAB
Streck Tissue Fixative	1% CTAB
	1% Sarkosyl
10% 1 Methyl 2-pyrrolidinone	1% Sarkosyl
100 mM phenylglyoxal	1% Sarkosyl
RNA Later	1% Sarkosyl
Streck Tissue Fixative	1% Sarkosyl
	1% CTACl
600 mM tetrabutyl ammonium	No detergent
800 mM tetramethyl	No detergent

Figure 6



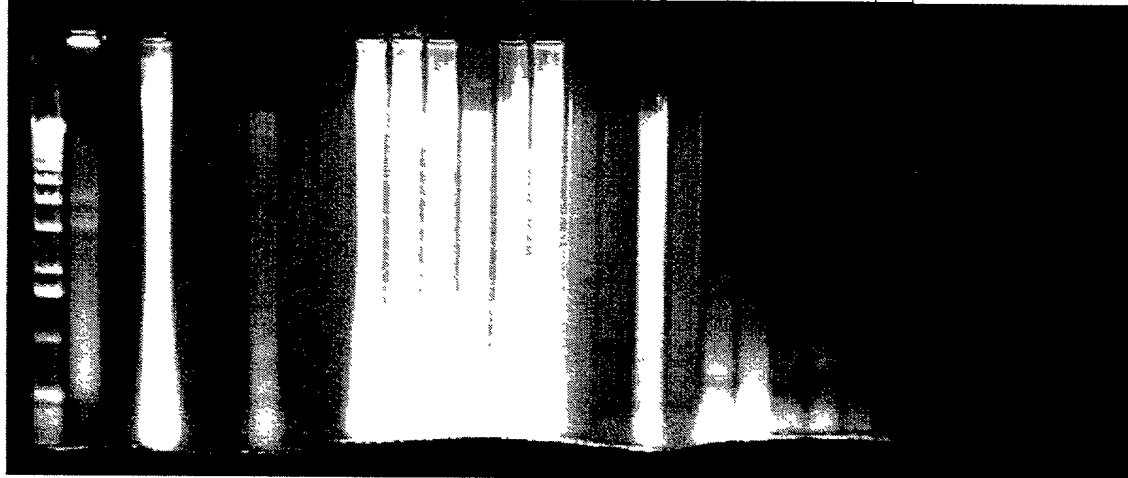
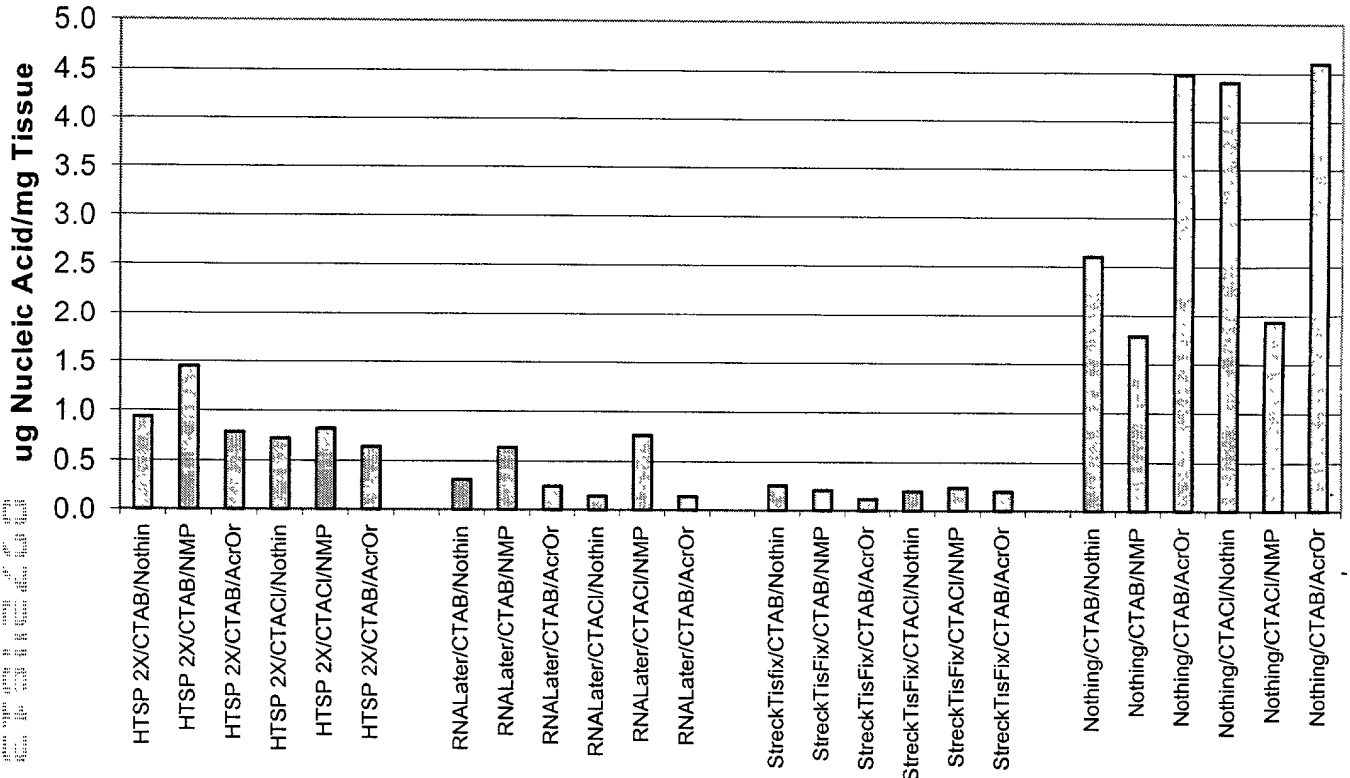


Figure 8

None	Cetyltrimethylammonium bromide	
phenylglyoxal	Cetyltrimethylammonium bromide	
1-methyl-2-pyrrolidinone	Cetyltrimethylammonium bromide	
Acridine Orange	Cetyltrimethylammonium bromide	
None	Cetyltrimethylammonium chloride	
phenylglyoxal	Cetyltrimethylammonium chloride	
1-methyl-2-pyrrolidinone	Cetyltrimethylammonium chloride	
Acridine Orange	Cetyltrimethylammonium chloride	
None	Hexadecyltrimethylammonium bromide	
phenylglyoxal	Hexadecyltrimethylammonium bromide	
1-methyl-2-pyrrolidinone	Hexadecyltrimethylammonium bromide	
Acridine Orange	Hexadecyltrimethylammonium bromide	
None	Hexadecyltrimethylammonium chloride	
phenylglyoxal	Hexadecyltrimethylammonium chloride	
1-methyl-2-pyrrolidinone	Hexadecyltrimethylammonium chloride	
Acridine Orange	Hexadecyltrimethylammonium chloride	
None	Sarkosyl	
phenylglyoxal	Sarkosyl	
1-methyl-2-pyrrolidinone	Sarkosyl	
Acridine Orange	Sarkosyl	
	No detergent	

# **Effect of Tissue Presoaking** **1 mg Proteinase K, 45°C 30 minutes**



**Figure 9**

		2XHTSP		RNA Later		Streck Tissue Fixat		Nothing	
		CTAB	CTACI	CTAB	CTACI	CTAB	CTACI	CTAB	CTACI
	Nothing								
	1-methyl-2-pyrrolidinone								
	Acridine Orange								
	Nothing								
	1-methyl-2-pyrrolidinone								
	Acridine Orange								
	Nothing								
	1-methyl-2-pyrrolidinone								
	Acridine Orange								
	Nothing								
	1-methyl-2-pyrrolidinone								
	Acridine Orange								
	Nothing								
	1-methyl-2-pyrrolidinone								
	Acridine Orange								
	Nothing								
	1-methyl-2-pyrrolidinone								
	Acridine Orange								

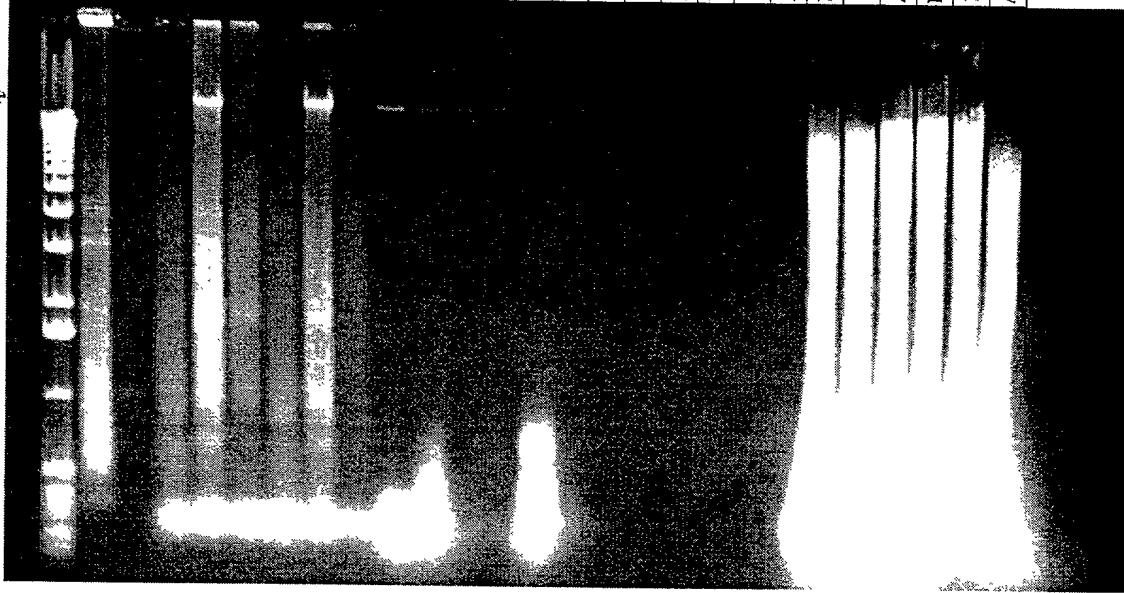
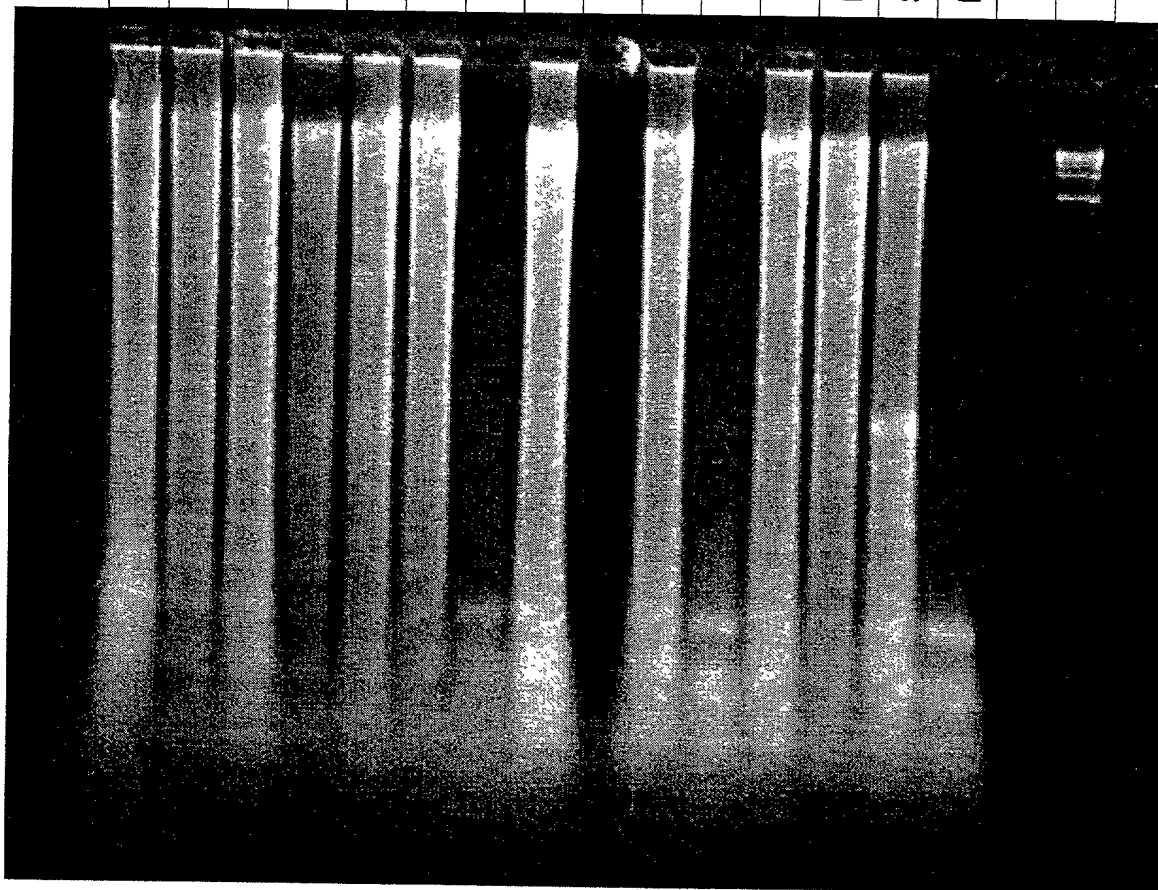


Figure 10



1% CTAB						1% CTACl						1% SDS					
5 mM Aurintricarboxylic Acid						5 mM Aurintricarboxylic Acid						5 mM Aurintricarboxylic Acid					
2 mM Aurintricarboxylic Acid						2 mM Aurintricarboxylic Acid						2 mM Aurintricarboxylic Acid					
1 mM Aurintricarboxylic Acid						1 mM Aurintricarboxylic Acid						1 mM Aurintricarboxylic Acid					
0.5 mM Aurintricarboxylic Acid						0.5 mM Aurintricarboxylic Acid						0.5 mM Aurintricarboxylic Acid					
0.2 mM Aurintricarboxylic Acid						0.2 mM Aurintricarboxylic Acid						0.2 mM Aurintricarboxylic Acid					
0.1 mM Aurintricarboxylic Acid						0.1 mM Aurintricarboxylic Acid						0.1 mM Aurintricarboxylic Acid					

Figure 11



Dodecyltrimethylammonium bromide
Tetradecyltrimethylammonium bromide
Cetyltrimethylammonium bromide
Cetyltrimethylammonium chloride
Hexadecyltrimethylammonium bromide
Hexadecyltrimethylammonium bromide
Mackernium 006 (Polyquaternium 6)
Mackernium KP (Olealkonium chloride)
Mackernium NLE (Quaternium-84)
Mackernium 007 (Polyquaternium-7)
Mackernium Stearalkonium SDC85 Chloride
Benzalkonium chloride
SDS
Nothing

Figure 12

## DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: COMPOSITIONS, METHODS, AND KITS FOR ISOLATING NUCLEIC ACIDS USING SURFACTANTS AND PROTEASES, the specification of which is attached.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 385(a) of any PCT International application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

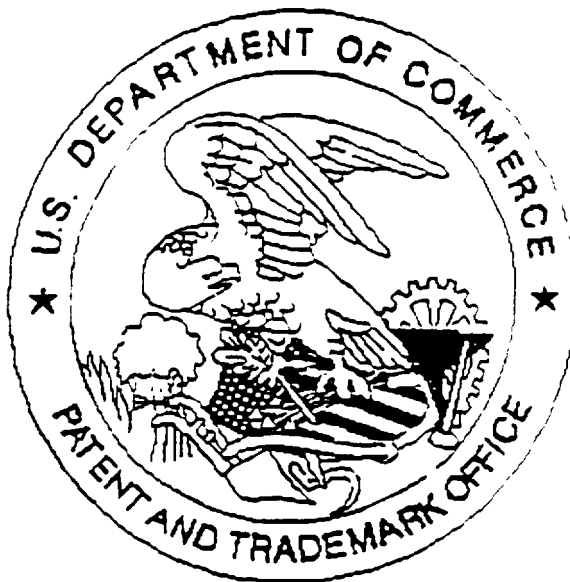
Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P., CUSTOMER NUMBER 22,852, Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Heffer, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,810; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,690; Dennis P. O'Reilly, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,519; Charles E. Lipsey, Reg. No. 28,185; Thomas W. Winland, Reg. No. 27,805; Basil J. Lewis, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,824; Susan Haberman Giffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,148; Carol P. Elinaudi, Reg. No. 32,220; Walter Y. Boyd, Jr., Reg. No. 31,738; Steven M. Anzalone, Reg. No. 32,095; Jean B. Fordis, Reg. No. 32,984; Barbara C. McCurdy, Reg. No. 32,120; James K. Hammond, Reg. No. 31,964; Richard V. Burgulian, Reg. No. 31,744; J. Michael Jakes, Reg. No. 32,824; Thomas W. Banks, Reg. No. 32,719; Christopher P. Isaac, Reg. No. 32,618; Bryan C. Diner, Reg. No. 32,408; M. Paul Barker, Reg. No. 32,013; Andrew Chanho Sonu, Reg. No. 33,457; David S. Forman, Reg. No. 33,694; Vincent P. Kovalick, Reg. No. 32,867; James W. Edmondson, Reg. No. 33,871; Michael R. McGurk, Reg. No. 32,045; Joann M. Neth, Reg. No. 36,363; Gerson S. Panitch, Reg. No. 33,751; Cheni M. Taylor, Reg. No. 33,216; Charles E. Van Horn, Reg. No. 40,288; Linda A. Wadler, Reg. No. 33,218; Jeffrey A. Berkowitz, Reg. No. 36,743; Michael R. Kelly, Reg. No. 33,921; James B. Monroe, Reg. No. 33,971; Doris Johnson Hines, Reg. No. 34,829; Allen R. Jensen, Reg. No. 26,224; Lori Ann Johnson, Reg. No. 34,498; and David A. Manspelzer, Reg. No. 37,540 and John W. Burns, Reg. No. 43,520. Please address all correspondence to FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P. 1300 I Street, N.W., Washington, D.C. 20005, Telephone No. (202) 408-4000.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full Name of First Inventor I. Lawrence Greenfield	Inventor's Signature <i>I. Lawrence Greenfield</i>	Date 11-28-00
Residence 242 Louise Lane, San Mateo, CA 94403		Citizenship USA
Post Office Address Same as above		

United States Patent & Trademark Office  
Office of Initial Patent Examination -- Scanning Division



SCANNED, #16

Application deficiencies were found during scanning:

☐ Page(s) \_\_\_\_\_ of \_\_\_\_\_ were not present:  
for scanning. (Document title)

☐ Page(s) \_\_\_\_\_ of \_\_\_\_\_ were not present:  
for scanning. (Document title)

☒ Scanned copy is best available. Drawings